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Identification and modulation of novel nicotinic acetylcholine receptors from parasitic nematodes

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Identification and modulation of novel nicotinic acetylcholine receptors from parasitic nematodes

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

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DEDICATION

I wholeheartedly dedicate this thesis to my loving and caring parents

Lekh Ram Choudhary and Nirmla Choudhary,

and to my dearly beloved husband, Swapnanjan Chatterjee.

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ABSTRACT

Parasitic nematodes, particularly soil transmitted helminths (STHs), are among the most frequent causes of physical and intellectual growth retardation in humans. Furthermore, parasitic nematodes of livestock and crops result in substantial economic losses, threatening the overall sustenance of the expanding population. Although rarely lethal, infections typically result in chronic, disabling, and disfiguring morbidity adversely affecting the essential components of human development. Chemotherapy continues to serve as the mainstay for treatment and control of nematode infections as there are no vaccines for human use. The global strategy for the control involves regular administration of anthelminthic drugs to the high risk-populations. STHs mainly effect resource limited and poverty-stricken populations. This segment of the population can neither afford conventional treatment nor improve their infrastructure for better sanitation and hygiene facilities. Furthermore, there is limited research investment in the anthelmintics market due to high costs and modest profit levels, which has slowed down the anthelmintic drug discovery over the years. In addition, the widespread resistance to the limited armamentarium of anthelmintic drugs in the veterinary field has raised concerns in human medicine as well. It is prudent to restore the depleted drug-discovery pipeline before the existing antiparasitic drugs are rendered unviable. Thus, research should be directed towards identification and development of novel antinematodal targets and new therapeutics.

We have described a novel nicotinic acetylcholine receptor formed by a non-α subunit, EAT-2, from the pharynx of *Caenorhabditis elegans*, a model nematode. We hypothesize that activation of this receptor will lead to pharyngeal paralysis similar to the

avermectins, which target glutamate-gated chloride ($GluCl^-$) channels in pharyngeal tissue. For the first time we find that a non-alpha nicotinic subunit can form a homomeric ligand gated ion-channel when expressed *in vitro*. Even though EAT-2 is most closely related to vertebrate α-7 nAChRs, its pharmacological profile is distinct from previously characterized vertebrate channels and nematode somatic muscle nAChRs. The pharyngeal receptor was not activated by many of the cholinergic anthelmintics (levamisole, tribendimidine, pyrantel) and was insensitive to α-bungarotoxin and dihydro-β-erythroidine (DHβE). We also characterized the homologous receptor from *Ascaris suum*, a model for human parasitic nematode *A. lumbricoides*. We investigated the *A. suum* pharynx using electrophysiology and identified a significant cholinergic component that was pharmacologically similar to the *A. suum* EAT-2 receptor expressed in oocytes. This receptor was not sensitive to existing cholinomimetic anthelmintics and therefore represents a promising drug target. Importantly, the EAT-2 nAChR from both of the nematode species requires a novel auxiliary protein, EAT-18, for functional expression. EAT-18 directly interacts with the non-α subunits and modifies the pharmacological profile of the receptor. To the best of our knowledge this is only the 2nd auxiliary protein described for nAChRs and the first report of an auxiliary protein that is essential for functional expression in any cys-loop ligand-gated ion channel (LGIC). Both EAT-2 and EAT-18 proteins from *A. suum* were expressed in tissues other than the pharynx suggesting the receptor may serve multiple physiological functions. Thus, we have identified a non-canonical homomeric channel formed by non-alpha nAChR subunits as a potential novel drug target and a new type of obligate auxiliary protein for nAChRs.

Secondly, we have identified an ACR-16 nAChR subunit homologue from *Ancylostoma caninum*, a model for human hookworm infections. *Aca*-ACR-16 forms a functional homomeric channel when expressed in *Xenopus laevis* oocytes. *A. suum* ACR-16 nAChRs have been studied previously and reported to be a suitable target for the development of therapeutic drugs. In this study we explored the effects of several cholinergic agonists and antagonists on the expressed *A. caninum* ACR-16 nAChR using two-electrode voltage-clamp. The pharmacological profile of the *Acn*-ACR-16 receptor showed some similarities to the *A. suum* ACR-16 nAChRs. Both the homologues were unresponsive to many of the existing cholinomimetic anthelmintics (levamisole, pyrantel, morantel, bephenium and tribendimidine). Like *Asu*-ACR-16, the *Aca*-ACR-16 nAChR was also highly sensitive to mecamylamine and d-TC; moderately sensitive to derquantel and hexamethonium. In addition to similarities, the *A. caninum* ACR-16 nAChR showed marked differences in pharmacological sensitives from *A. suum* homologue which makes it scientifically interesting. As opposed to *Asu*-ACR-16, 3-Bromocytisine was the most potent agonist of *Aca*-ACR-16 while oxantel failed to activate the *A. caninum* nAChR. The mean time constants of agonists for desensitization rates were longer for *Acn*-ACR-16 (between 6.2 and 12.6s) in comparison to *Asu*-ACR-16 (between 1.5 and 4.8s). In contrast to *Asu*-ACR-16, the *A. caninum* receptor was completely inhibited by DhβE and moderately inhibited by α-BTX. In conclusion, we have successfully recapitulated a fully functional homomeric ACR-16 nAChR from *A. caninum*. The pharmacology of the receptor is distinct from other somatic muscle nematode receptors. The ACR-16 homologue from *A. caninum* also displayed some pharmacological differences from *Asu*-

ACR-16. Therefore, we suggest that *A. caninum* ACR-16 may be a valid target site that should be further exploited for the development of agents against hookworm infection.

Lastly, we have shown that the monoterpenoid compounds, menthol and carvacrol, can be potentially used for antinematodal therapy in combination with existing cholinergic compounds. We examined the effects of twelve monoterpenoid phytocompounds on a heterologously expressed levamisole sensitive nAChR from *Oesophagostomum dentatum* and a nicotine sensitive nAChR from *A. suum* using the two-electrode voltage-clamp technique. The majority of these compounds acted as antagonists when tested at 100 μM concentration. Carvacrol produced significant non-competitive inhibition suggesting the compound acts at site different from the ligand binding sites. This monoterpenoid phytocompound has also been shown to target GABA (gamma amino-butyric acid) and tyramine receptors in previous studies. This multifaceted polypharmacological effect of carvacrol might make it efficacious as an anthelmintic, alone or in combination, and help circumvent the development of resistance. Interestingly, menthol potentiated the amplitude of acetylcholine currents in our antagonist experiments. Further investigation revealed that both 0.1 μM and 10 μM menthol potentiated acetylcholine and levamisole mediated responses in the levamisole sensitive nAChR. We also investigated the effects of 0.1 μM menthol on the contractility of *A. suum* somatic muscle strips. Menthol significantly potentiated the peak contractions at each concentration of acetylcholine tested. We have provided promising evidence of positive allosteric modulation by menthol in both *in vivo* and *in vitro* experiments. Thus, menthol could possibly be used in combination therapy with cholinomimetic drugs like pyrantel or levamisole to produce a more potent anthelmintic effect. In summary,

menthol and carvacrol can contribute to antinematodal phytotherapy and alleviate the pressure on the limited classes of antiparasitic agents available.

CHAPTER 1. GENERAL INTRODUCTION

1.1 Introduction

Nematodes are highly evolved metazoan organisms with ubiquitous existence. They exhibit complex physiological behaviors, including locomotion, navigation, feeding and reproduction, that are regulated by a sophisticated neuromuscular system. Parasitic nematodes are dependent on these behaviors for survival in the host and the environment. These biological functions are often targeted through chemotherapy to kill them or disrupt their life cycle. There are three major classes of anthelmintic drugs: the benzimidazoles (BZs), macrocyclic lactones (MLs), and imidazothiazoles/ tetrahydropyrimidines. The benzimidazoles such as thiabendazole, mebendazole, and albendazole inhibit microtubule synthesis by targeting β-tubulin. Underlying nematode neuromuscular system is a large repertoire of ion channels, including nicotinic acetylcholine receptors (nAChRs) and glutamate-gated chloride channels (GluCls). These ionotropic channels integrate with the normal biological functions to maintain viability. GluCls are target site of macrocyclic lactones (e.g. ivermectin, abamectin) while imidazothiazoles/tetrahydropyrimidines (e.g. levamisole, pyrantel, oxantel) act on nicotinic acetylcholine receptors. nAChRs are evolutionarily conserved membrane-spanning protein complexes that are required for fast excitatory chemical-to-electrical transduction in nematodes. Many of these cation-selective receptors are localized to neuromuscular junction and are prime loci for fundamental cellular functions. Nematodes have a greater number of nAChR subunits than mammals. By varying the subunit composition and stoichiometry different pharmacological sensitivities can be achieved, which is a desired feature in drug discovery. Nematode nAChRs, especially those found

on muscles, hold emblematic position as anthelmintic targets for treatment of soil transmitted helminth (STH) infections. There are topographical differences in binding sites between nematodes and vertebrates, making these channels “druggable”. Cholinergic anthelmintics, including levamisole, pyrantel and more recently introduced monepantel cause prolonged activation of excitatory nAChRs in nematodes leading to rapid immobilization and expulsion from the host. STHs infect about 1.5 billion people worldwide and hamper socioeconomic development through human debility and by decimating livestock and crop production. Chemotherapy has brought manifold improvements in health, agricultural economics, livestock production and overall food security. Nevertheless, repeated mass drug administration of monotherapies with limited classes of antiparasitic agents has driven widespread resistance in veterinary anthelmintics. There are reports of reduced cure rates in humans and increased risk of development of drug resistance. Furthermore, there are no vaccines for humans use. There is an accepted need for discovery and development of novel chemotherapeutic targets as well new therapeutics.

These doctoral studies were undertaken to address strategies that can circumvent the issue of drug resistance and strengthen the future defense against nematode infections. The first aim was to characterize the pharmacology of a novel nAChR, EAT-2, from free-living (*Caenorhabditis elegans*) and parasitic (*Ascaris suum*) nematodes. We also identified a novel auxiliary subunit for EAT-2 cation selective channels, EAT-18, which is an essential element of the receptor complex and modulates its pharmacology. The second aim was to validate ACR-16 nAChR from *Ancylostoma caninum*, a laboratory model for the human parasite, as a target site for the development of

anthelmintics against hookworm infections. The third aim was to evaluate the potential anthelmintic effects of monoterpenoid compounds to identify alternatives to current synthetic nematocidal agents.

1.2 Thesis organization

In this thesis, Chapter-1 provides a general introduction about parasitic nematodes and the importance of nicotinic acetylcholine receptors as chemotherapeutic targets. In view of widespread drug resistance in veterinary anthelmintics and the risk of development of resistance in human medicine, it is prudent to develop novel strategies for sustainable control of parasitic infections in foreseeable future. We have highlighted the importance of characterization of new drug targets and development of phytotherapeutics as anthelmintic agents as means of addressing drug resistance concerns. In Chapter-2, I have reviewed the literature pertaining to parasitic nematodes and associated infections, particularly ascariasis, hookworm infections and oesophagostomiasis, nicotinic acetylcholine receptors (nAChRs), auxiliary protein subunits of nematodes, *C. elegans* and *A. suum* pharynx and monoterpenoid compounds. In Chapters 3, 4 and 5, I present research work done by myself and collaborators that has been published (Chapter 5) or in preparation for publication (Chapter 3 and 4). Chapter 3 describes the pharmacological characterization of a novel homomeric pharyngeal nAChR formed by a non-alpha subunit, EAT-2, from *C. elegans* (free-living nematode) and *A. suum* (nematode model for human ascariasis). EAT-2 requires a small single-pass novel auxiliary protein, EAT-18, for successful heterologous expression. Both the proteins may be used for potential targeted chemotherapy through development of selective anthelmintic drugs. I did all the

work outlined in this paper, except *in vivo* electrophysiology recordings from *A. suum* pharynx and isolation of cytoplasm for single-cell PCR which were done in our lab. Chapter 4 describes the cloning and *in vitro* expression of ACR-16 nAChRs from *A. caninum*, a clade V nematode parasite. We generated a comparative pharmacological analysis of the homomeric channel and established ACR-16 as a potential target in the hookworm parasites. All the work in this chapter was done by me with input from our collaborators at the Iowa State University. Chapter 5 describes the potential of monoterpenoid compounds as anthelmintic compounds. We identified menthol as a promising candidate for combination therapy with cholinergic anthelmintics. I did most of the work presented in this paper. The cloning of the receptor subunits was done by a former graduate student in our lab and the *A. suum* muscle flap contraction studies were conducted by our collaborators from Belgrade. Chapter 6 is a general discussion and conclusions of my PhD research and suggestions for future work. Appendix A is an invited review on advances in anthelmintic drug development and Appendix B is a list of all conferences, abstracts and awards received.

CHAPTER 2. LITERATURE REVIEW

2.1 Soil-transmitted helminth (STH) infections

Helminth parasites have accompanied humans throughout the ages and continue to be a nuisance especially in regions where poverty persists. The term helminth is derived from Greek word meaning “worm”. Primitive civilizations had knowledge of parasites and parasitic infections to a certain extent. Many ancient texts provide abundant evidence and detailed descriptions of infections that clearly support the presence of helminthic manifestations. Ebers papyrus (1500 B.C.), the Egyptian medical record mentions worms, which most certainly are *Ascaris lumbricoides* and the Guinea-worm, *Dracunculus medinensis* (Hoeppli 1956). Many of the characteristic clinical features of helminth infections are described in historical records of Arabic physicians, particularly Rhazes (AD 850 to 923) and Avicenna (AD 980 to 1037), Roman physicians from 700 BC to 400 AD, Chinese writings from the 2nd and 3rd century BC and written records by Hippocrates, known as the *Corpus Hippocratorum* (Hoeppli 1956; Jones and Whithington 1948-1953; Grove 1990). There is mention of the “fiery serpents” in the Bible that struck down the Israelites in the region of the Red Sea after the Exodus from Egypt somewhere about 1250 to 1200 BC (Cox 2002); most parasitologists believe these were Guinea worms. A condition interpreted as dracunculiasis has also been described in the Assyrian texts present in the library of King Ashurbanipal from the 7th century BC (Hoeppli 1959). *A. lumbricoides* eggs have been found in coprolites from pre-Hispanic and pre-Columbian Peruvian populations dating back to 2277 B.C. (Horne 1985; Patrucco, Tello, and Bonavia 1983) and Brazilian populations around 1660-1420 BC (Ferreira, de Araujo, and Confalonieri 1980, 1983). Records have also confirmed *A.*

lumbricoides infestation in an Egyptian mummy dating between 1938-1600 BC (Cockburn, Cockburn, and Reyman 1998) and an ancient Western Han Dynasty corpse from 1368-1644 AD (Wei et al. 1981). Fossilized ova of *Ancylostoma* sp. have been found in human feces dating somewhere between 3350 BC and 480 AD (Ferreira et al. 1987). Paleo-parasitological investigations have established that the people of the Korean Joseon Dynasty were also heavily infected by helminth parasites (Ki, Bae, and Shin 2013). The Babylonian Talmud (4th to 5th century AD) mentions the common human parasites and contains highly developed hygiene laws (Hoeppli 1956). The ancient Indian text, Atharvaveda, also contains references to parasites and corresponding infections; some of these have been interpreted to be the intestinal helminths, *A. lumbricoides* and *Enterobius vermicularis* (Jolly and Kashikar 1951). Thus, the history of helminth parasitology is fascinating. Many of the important parasites encountered today had a widespread existence and our ancestors were aware of their manifestations. The current trend of helminths and corresponding infections resulting from their interaction with humans is still an enduring issue, influenced by socio-cultural spectrum (Alum, Rubino, and Ijaz 2010).

Helminths are a diverse group of metazoan organisms that include both parasitic and free-living worms. Humans are hosts to nearly 341 helminth species, but only 25 of those produce sufficient adverse impact to be considered as globally significant (Coombs and Crompton 1992). These worms are highly evolved, opportunistic organisms and are responsible for the most common parasite infections (Lindquist and Cross 2017; Cleaveland, Laurenson, and Taylor 2001; Jones et al. 2008; Awasthi, Bundy, and Savioli 2003). The helminth worms are divided into four phyla, namely, Platyhelminthes

(tapeworms and trematodes), Nematoda (roundworms or nematodes), Acanthocephala (spiny-headed worms), and Nematophora (hairworms); only worms belonging to the first two are endoparasites of humans (Despommier et al. 2017). The phylum Nematoda is a diverse group of helminths, characterized by species richness, biodiversity, abundance and ecological omnipresence (Dieterich and Sommer 2009; Blaxter et al. 1998). The word Nematoda comes from the Greek words "nematos", meaning thread, and "eidos", meaning form. More than 25,000 nematode species have been described in literature, including nearly 16,000 parasitic species, with new species still being identified and recorded (Dieterich and Sommer 2009; Okulewicz, Perec, and Hildebrand 2005). Parasitic nematodes can infect a wide spectrum of hosts and threaten the health of humans, animals and plants on a global scale. These include soil-transmitted helminths (STHs) that affect over 1.5 billion people worldwide (WHO 2019). These are also known as soil-transmitted nematodes (STN) or geohelminths. They share a common transmission mechanism involving contact with infective parasitic eggs or larva through exposure to fecally contaminated water, soil or contaminated food. The predominant species involved in STH infections are: *Ascaris lumbricoides* (roundworm), *Necator americanus* and *Ancylostoma duodenale* (hookworms), *Trichuris trichiura* (whipworm) and *Strongyloides stercoralis* (the threadworm). STH infections are among the major contributors to the global burden of infections and are endemic in more than half of the world's countries (Figure 2.0) (WHO 2017a, 2017b). Ascariasis is reported in 771.7–891.6 million people, while whipworms infect around 429.6–508.0 million people and an estimated 406.3–480.2 million are infected with hookworms (Jourdan et al. 2018; Vos et al. 2017). The populations living in resource

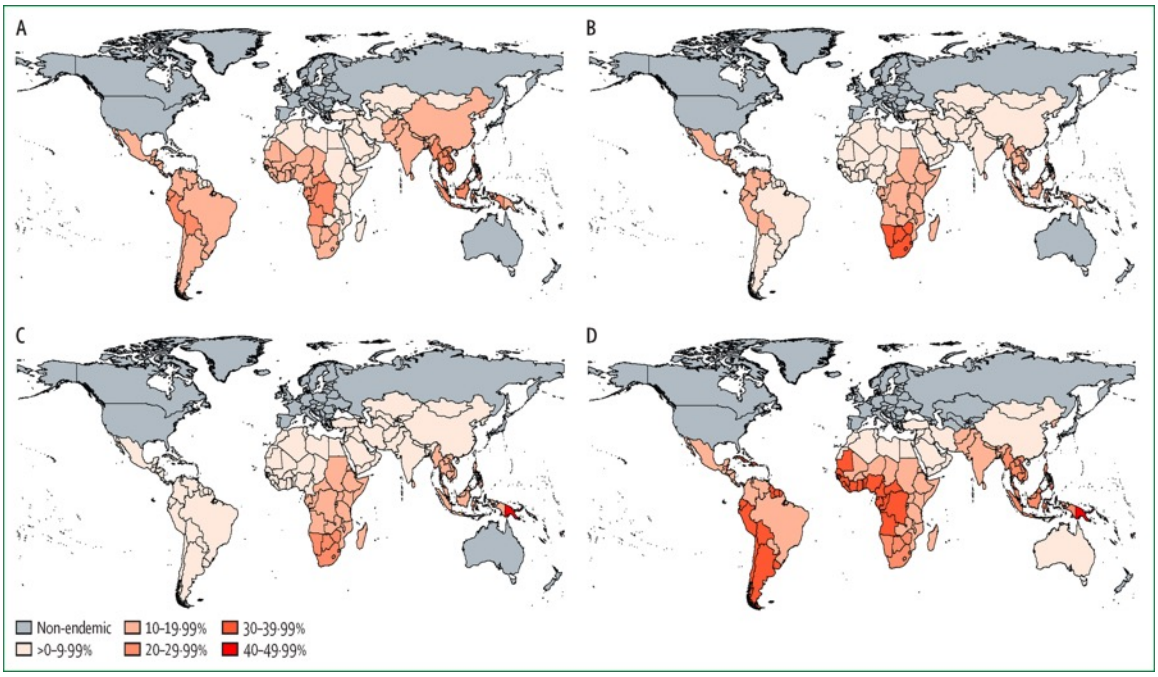

Figure 2.0 Global prevalence of various species of STH worms (Jourdan et al. 2018). A. *Ascaris lumbricoides* (2010). B. *Trichuris trichiura* (2010). C. *Necator americanus* and *Ancylostoma duodenale* (for 2010). D. *Strongyloides stercoralis* (2011).

limited and poverty-stricken regions of sub-Saharan Africa, Asia and the Americas, are infected with at least one species of STH (Hotez et al. 2007; Hotez et al. 2006; WHO 2019; Stolk et al. 2016). Heavy burdens of STH worms are associated with gastrointestinal manifestations (abdominal pain, diarrhea, blood and protein loss, rectal prolapse), pruritis, anemia and malnutrition through loss of blood and nutrients (Bethony et al. 2006). Children are particularly susceptible to these geohelimths and in many cases they harbor all three types of STH (hence the moniker “the unholy trinity”) (Despommier et al. 2017). Childhood malnutrition can have profound impact on the physical development and cause cognitive deficits impacting educational attainment and future productivity (WHO 2019; Bethony et al. 2006). STH infections perpetuate a persistent drain on social-economic development of low-income regions of the world (King 2010). Soil transmitted nematodes also infect a wide range of animal and plant species,

representing a significant economic and welfare burden and a substantial threat to global food security.

2.1.1 Ascariasis

A. lumbricoides is a large roundworm responsible for causing a spectrum of disease symptoms known as ascariasis in humans. It is one of the most common parasites in the world and infects around 800 million people worldwide (Vos et al. 2017). It is responsible for around 1 million DALYs (Disability adjusted life years; measure of overall disease burden, expressed as the number of years lost due to ill-health, disability or early death) with more than 60,000 deaths annually (Shah and Shahidullah 2018; de Vlas et al. 2016). The intensity and frequency of infections with *A. lumbricoides* is highest in children aged 5-15 years (Bethony et al. 2006; Hotez et al. 2008). *Ascaris suum*, which causes ascariasis in pigs, can occasionally cause infection in humans through contact with domestic pigs (Nejsum et al. 2012; Leles et al. 2012). *A. lumbricoides* has a widespread distribution in temperate and subtropical regions with the highest prevalence in rural populations of tropical zones, where hygiene and sanitation are inadequate. The warm and humid climate in these regions favors year-round transmission of infection (Shah and Shahidullah 2018; Pullan et al. 2014).

Humans are the definitive hosts in the *A. lumbricoides* lifecycle (Figure 2.1) and the individuals are infected through fecal-oral transmission (Despommier et al. 2017; Dold and Holland 2011). After the infective eggs are swallowed, first-stage larvae (L1) hatch in the jejunum and molt into second-stage larvae (L2). These L2 larvae then penetrate the intestinal mucosa and reach the liver via the portal blood, where the L2 cuticle is shed. Following migration to the liver, the larvae travel to the pulmonary circulation. In

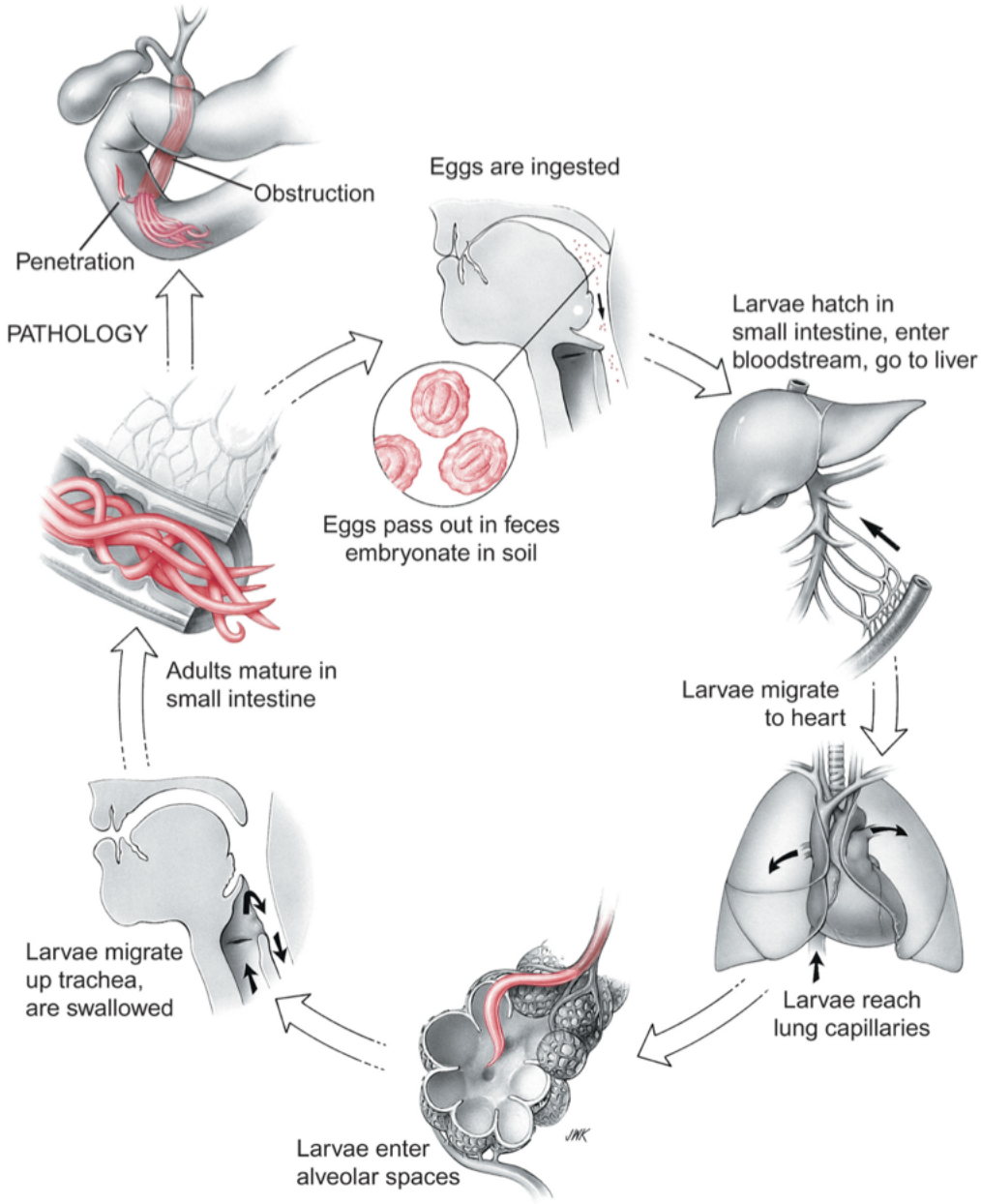

Figure 2.1 Lifecycle of *Ascaris lumbricoides* (Despommier et al. 2017).

the capillaries of the lungs, they grow bigger and molt twice. The developed larvae break through the capillaries, move across the alveolar wall and ascend the tracheobronchial tree to the larynx. They are swallowed into the intestine where they molt and mature into adults. Mature male and female worms measure 15-31 cm and 20-35 cm, respectively. The adult worms have a lifespan of upto 1 to 2 years, with mature females producing

thousands of fertilized or unfertilized eggs daily, that are passed in the stool (Sinniah 1982). The time period between infection and egg production is usually 2-3 months. Unembryonated eggs are very durable and remain viable in warm, moist soil for years.

The majority of infections with *A. lumbricoides* are asymptomatic and pathology of the disease is largely related to worm burden (De Silva, Guyatt, and Bundy 1997). When symptoms do occur, they can be mild such as abdominal discomfort to heavy such as diarrhea, anemia, malnutrition leading to impaired physical and mental growth in children (Bethony et al. 2006; Crompton 2001). The major serious sequalae of heavy worm burden is intestinal obstruction, volvulus or intussusception which can be fatal in upto 6% of cases (Pullan et al. 2014; Bethony et al. 2006; Tietze and Tietze 1991). The migration of worms through the body can lead to coughing and sometimes coughing up of worms and wheezing (Roberts and Kemp 2001; Dold and Holland 2011). Obstruction of the gall bladder can result in cholangitis, cholecystitis, biliary colic, liver abscess, and pancreatitis (Roberts and Kemp 2001). *A. lumbricoides* can cause type-1 hypersensitivity reactions to the larval stages and also induce a T-helper-2 cell (Th2) immune response (Jarrett and Miller 1982; Fitzsimmons, Falcone, and Dunne 2014; Akuthota and Weller 2012).

The diagnosis of ascariasis is usually made by microscopic examination of fecal samples for the presence of eggs. Visualization and description of the worms can also establish identification (Knopp et al. 2008; Nikolay, Brooker, and Pullan 2014; Bethony et al. 2006). Treatment of ascariasis includes administration of a single dose of albendazole (400 mg) or mebendazole (500 mg) (WHO 2019; Claus et al. 2018; Keiser and Utzinger 2008; Albonico, Crompton, and Savioli 1999).

2.1.2 Hookworm infections

Adult hookworms of the two species, *Necator americanus* and *Ancylostoma duodenale*, account for most infections in humans, parasitizing the upper part of the small intestine. Hookworm infections are common throughout sub-Saharan Africa, tropical regions of the Americas, South China and Southeast Asia (Bethony et al. 2006; de Silva et al. 2003b; Hotez et al. 2004). *N. americanus* is the more predominant hookworm worldwide, except in some focal areas of India, China and Egypt (Despommier et al. 2017). *Ancylostoma ceylanicum*, hookworm species of humans and other mammals, is found in Southeast Asia but is not considered an important pathogen (Ngui et al. 2012). The canine hookworm species, *A. caninum*, can cause zoonotic infections resulting in enteritis, localized myositis and cutaneous larva migrans (Landmann and Prociv 2003; Prociv and Croese 1996; Traversa 2012). *A. braziliense*, hookworm of dogs and cats, can also penetrate the human skin causing cutaneous larva migrans (Blackwell and Vega-Lopez 2001). An estimated 500 million people are infected by hookworms and the associated infections account for >4 million disability adjusted life years (DALYs) lost annually (Loukas et al. 2016; Vos et al. 2017; Bartsch et al. 2016).

The percutaneous lifecycle of hookworm, *N. americanus*, is shown in Figure 2.2 (Despommier et al. 2017). The larvae of *A. duodenale* and *N. americanus* are free-living in the soil. The infection begins when the filariform L3 larval stage actively penetrates the cutaneous tissue of the skin usually through hair follicles or an abraded area. The filariform larvae are carried passively to the lung capillaries through the bloodstream. The larvae then penetrate the alveolar wall, crawl up the bronchi and trachea and pass to the larynx. They are swallowed and proceed into the small intestine. The larvae molt

twice resulting into development of mature adult worms over a period of 1-2 months. The adult worms have an average life span of up to one year for *A. duodenale* and 3-5 years in case of *N. americanus* (Hoagland and Schad 1978). Adult female hookworms, similar to roundworms, can produce thousands of eggs daily which are released in the stool. The released eggs take 5-10 days to hatch in warm, moist, sandy soil or in feces. The hatched larvae (L1 or rhabditiform larvae) molt into L3, the infective stage larvae, that can survive for weeks. The *A. duodenale* are also infective through oral ingestion. Orally ingested larvae undergo two molts without leaving the intestinal tract of the host. This results in a syndrome known as Wakana disease, characterized by nausea, vomiting, pharyngeal irritation cough and dyspnea (Kojima 1999).

The blood feeding hookworms do not directly account for substantial mortality; instead the major clinical manifestations of hookworm infection are the consequences of chronic intestinal blood loss (Loukas et al. 2016; Hotez et al. 2004). Depending on the stage of infection, route of transmission and degree of worm burden, the hookworm infections lead to four potential manifestations, dermatitis, pneumonia, abdominal pain, and chronic iron deficiency anemia. Larval penetration through skin, often through the feet of barefooted individuals, may result in ground itch accompanied by intense pruritic, tortuous vesicular lesions due to larval migration. The amount of blood loss through the intestine of the host is dependent on the species of the worm as *A. duodenale* results in greater blood loss (Albonico et al. 1998). In contrast to ascariasis, there is a steady rise in prevalence and intensity of hookworm infections with age (Bethony et al. 2002). The pattern of these infections is also dependent on sex of the person as the prevalence and intensity is higher in males. Although, women and children are more vulnerable to

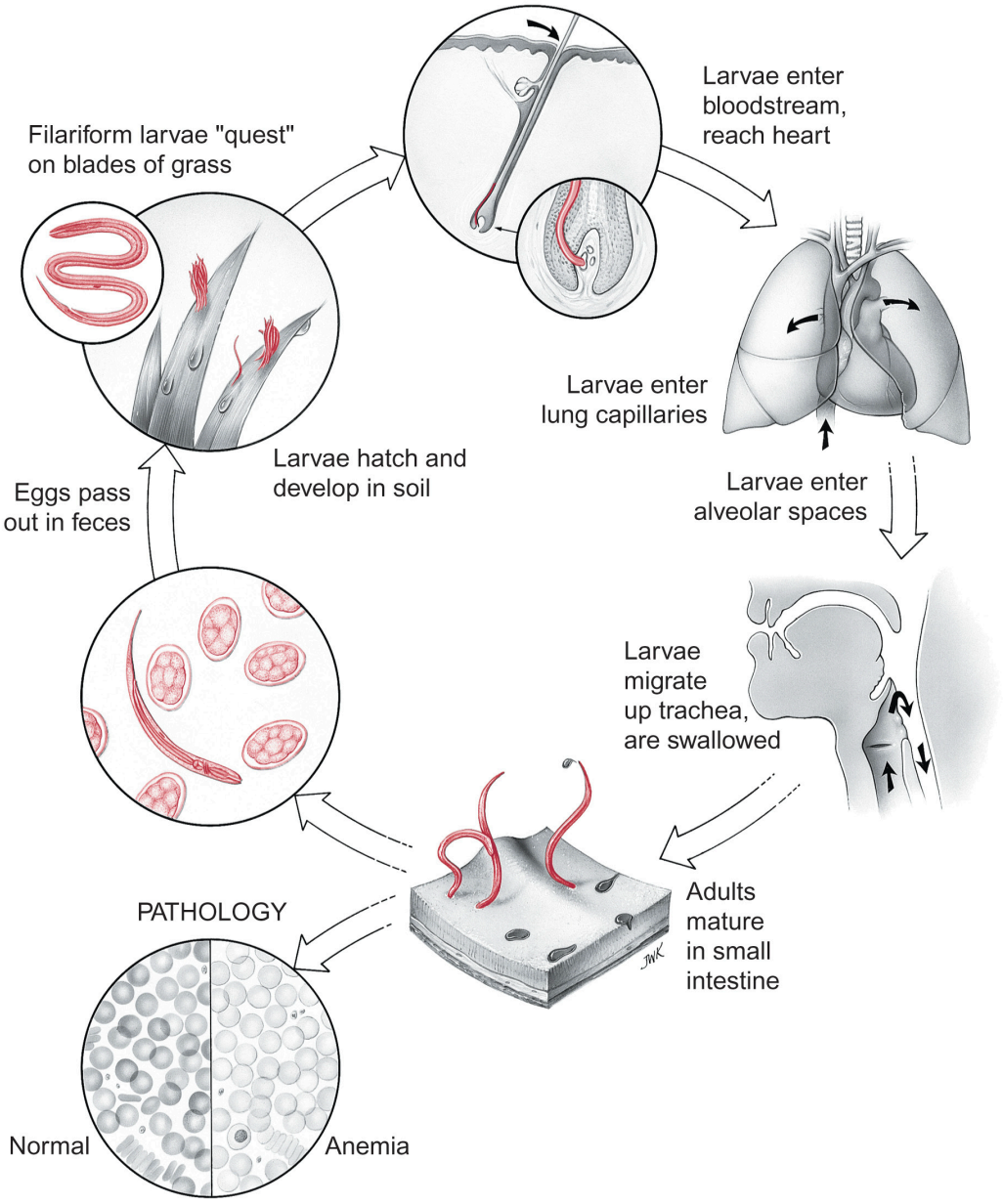

Figure 2.2 Lifecycle of *Necator americanus* (Despommier et al. 2017).

chronic blood loss as they have lower iron stores (Stoltzfus, Chwaya, et al. 1997; Stoltzfus, Dreyfuss, et al. 1997). Heavy infections in children lead to iron-deficiency anemia which in turn results in retarded physical growth and behavioral deficits (Hotez 2008; Loukas et al. 2016; Hotez et al. 2004; Despommier et al. 2017). Hookworm infections in adolescent girls and women of reproductive age adversely affect intrauterine

growth, birth weight and increases the likelihood of premature birth; in severe cases infections may contribute to maternal mortality (Hotez et al. 2008; Loukas et al. 2016; Brooker, Hotez, and Bundy 2008; Bethony et al. 2006; Bundy, Chan, and Savioli 1995).

Stool microscopy is the standard method for diagnosing the presence of hookworm by identifying hookworm eggs (Jourdan et al. 2018). In some cases, capsule endoscopy can be used (Wu, Chen, et al. 2016). A single dose of a benzimidazole anthelmintic, either albendazole (400 mg) or mebendazole (500 mg) is recommended for treatment. Pyrantel pamoate and levamisole are regarded as alternative drugs for the treatment of hookworm. Iron supplements and additional nutritional support is used in severe cases (Bethony et al. 2006; Keiser and Utzinger 2008; Sacko et al. 1999).

2.1.3 Oesophagostomiasis

The disease, oesophagostomiasis, is caused by species of roundworms belonging to the genus *Oesophagostomum*, falling under the family Strongylidae. The adult worms inhabit the lumen of caecum and colon in the host. The infection is characterized by the presence of nodular abscesses in the intestinal wall caused by the tissue-dwelling larval stage; hence the parasite is also known as the “nodular worm”. Most species of the oesophagostomes are parasitic in primates, with a few infecting swine, and ruminants (Gutierrez 2011). In humans most of the cases of oesophagostomiasis are normally considered as zoonotic infections, with the exception of *O. bifurcum* which does not require an animal reservoir (Polderman et al. 1991). *O. bifurcum* is the most common species infecting humans in Africa (Ghai et al. 2014; Despommier et al. 2017). The infection is focally endemic in northern Ghana and Togo with an estimated 250,000 cases, infecting up to 30% of the population (Storey et al. 2001; Ziem et al. 2006; Bogers

et al. 2001; Polderman et al. 1991; Polderman and Blotkamp 1995). Sporadic cases have been reported from parts of Asia and South America as well (Ziem et al. 2006).

The adult worms are roughly 1-2 cm long. Figure 2.3 shows the life cycle of *Oesophagostomum* spp. Mature females produce about 5,000 eggs daily, which are passed in the feces (Krepel and Polderman 1992). These eggs develop into L1 (first-stage) larvae when the temperature is conducive. L1 larvae feed on bacteria in the environment and molts twice to hatch into mature infective L3 (third-stage) larvae. The infective larvae are ingested by the host which subsequently invade the mucosa and sub-mucosa of the large intestine. At this stage distinct nodules can be recognized in the intestinal wall. After a few days, the larvae develop to fourth stage (L4) and escape the nodules to return to the lumen of the large intestine. The development of the L4 larvae into adult worms takes place in the lumen of large intestine (caecum and colon) (Anderson 1992; Despommier et al. 2017; Blotkamp et al. 1993). Egg production usually starts about 30 days post-infection.

Patients with oesophagostomiasis do not exhibit a definitive set of signs and symptoms. *O. bifurcum* infection can result in development of either a single nodular mass or a multi-nodular disease (Gigase et al. 1987; Storey et al. 2000). The uni-nodular disease, often referred to as Dapaong tumor, is the more predominant form of oesophaostomiasis, affecting 85% of patients. It presents as a palpable and painful protruding mass (30-60 mm in diameter) adherent to the abdominal wall, formed as a result of intense tissue reactions around a nodule containing single or a small cluster of encapsulated juvenile worms. This nodule can instigate peritonitis if ruptured and result in bowel obstruction, formation of cutaneous abscesses and fistulas through intense

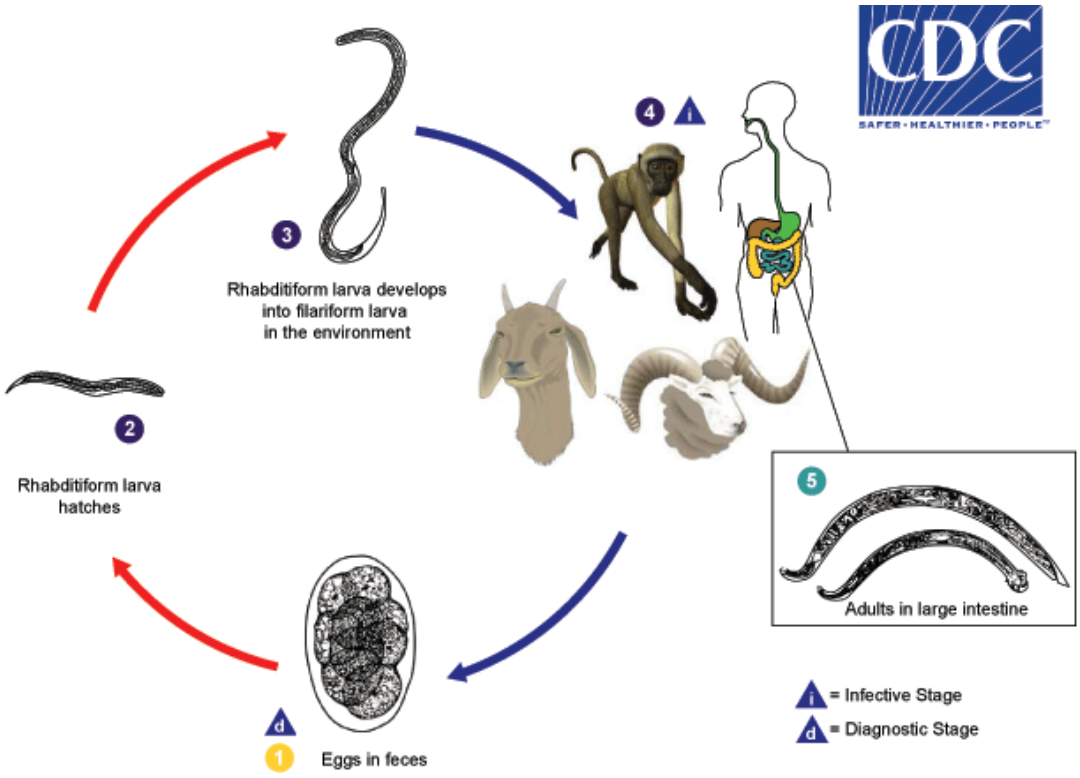

Figure 2.3 Lifecycle of *Oesophagostomum* spp. Modified from https://www.cdc.gov/dpdx/oesophagostomiasis/index.html (Accessed on 04-01-19 at 4:48PM CST).

secondary tissue reaction. The less common, multi-nodular oesophagostomiasis is characterized by the formation of hundreds of pea sized nodules containing worms and pus along the colon wall. This form of the infection can give rise to bowel obstruction, peritonitis, and intestinal volvulus (Polderman et al. 2010). Prevalence rate is higher in children belonging to ages 2-10, and females older than 5 years of ages. Although, male patients have larger sized nodules than females (Storey et al. 2001).

Microscopic examination of fecal samples is the diagnostic method of choice. Ultrasound and PCR amplification of DNA from fecal samples are also helpful for diagnosis and epidemiological studies (Storey et al. 2002; Verweij et al. 2001). Pyrantel pamoate and albendazole are used for treating infections due to *O. bifurcum* (Ziem et al.

2004). Surgical excision of the nodules is sometimes necessary for management of the infections (Storey et al. 2000).

2.2 Nematode body structure

The nematodes are cylindrical, bilaterally symmetrical, non-segmented roundworms that typically lack external appendages. Most of the nematodes are vermiform with tapering anterior and posterior ends. The phrase “tube within a tube” is used to describe their body structure as they possess a spacious fluid filled cavity that separates the outer body wall from the alimentary canal (Brusca and Brusca 1990; Gardner 2013). This cavity is not a true coelom as it is not completely lined with mesodermally derived cells and lacks a cellular lining or peritoneum, hence nematodes are called “pseudocoelomates”. The pseudocoelom consists of two to six fixed cells (celomocytes) and is mostly filled with intestinal and reproductive tissues. The pseudocoelomic fluid serves as a distributing medium for digested food and for collection of waste products (Chandler 1949; Brusca and Brusca 1990). It is under a positive pressure, relative to their environment, and assists with maintaining the worm’s body form. The body wall is composed of an outer cuticle and an inner layer of longitudinal somatic cells separated by a thin sheet of hypodermis (Lee 1965; Bird 1991). These layers appear as three concentric rings in a transverse section of the worms (Figure 2.4C).

The cuticle is a translucent non-cellular flexible outer covering, secreted by the underlying hypodermis that surrounds the entire body of the worms (Chandler 1949; Despommier et al. 2017; Castro 1996). It is primarily proteinaceous in composition with trace amounts of lipid and carbohydrates (Fetterer and Rhoads 1993). It lines the buccal cavity, esophagus, anus, cloaca, excretory pore and vagina. The cuticle can have different

forms; it may be smooth or ornamented with rings, longitudinal striations, or spikes. In some nematodes, the cuticle may have well-developed protruding fin-like projections either in the neck region, called cervical alae, in others in the tail region of the males supported by fleshy papillae called caudal alae (Gardner 2013). Another variability of cuticular complexes, known as bursa, are present on the posterior end of the male nematodes of some species. These are flaplike extension of the cuticle are used to grasp the female during copulation (Chandler 1949). The cuticle is structurally rigid to maintain the integrity of the worms, but flexible enough to permit bending and stretching. It serves as an interface connecting nematodes with their host. The cuticle is extremely resistant and protects the worms from getting digested in the inhospitable stomach environment of the vertebrate hosts. It is also involved in active transport of small molecules, including water, electrolytes, and organic compounds. During the worm's life-cycle, the cuticle is shed periodically, usually four times, before reaching the adult stage (Castro 1996; Chandler 1949; Despommier et al. 2017; Basyoni and Rizk 2016).

The cellular protoplasmic syncytial layer underlying the cuticle is the hypodermis. The nuclei of this subcuticular layer are present in four thickened chords, a dorsal, a ventral, and two lateral chords that divide the pseudocoelomic cavity into quadrants. Excretory tubules are located in the lateral chord while longitudinal nerve fibers are associated with the dorsal and ventral chords. The primary function of the hypodermis is to secrete the cuticle. It is also involved in the active exchange of water and small molecules (Fetterer and Wasiuta 1987; Gardner 2013; Poinar 2015; Chandler 1949).

The longitudinally arranged spindle-shaped somatic muscle cells are grouped into quadrants between the hypodermal chords and interdigitate with each other (Castro

1996; Chandler 1949; Stretton 1976). The somatic musculature forms the innermost layer of the body wall and surrounds the perienteric cavity. Each muscle cell consists of a contractile part with myofibrils and a non-contractile part (cell body) in which the nucleus, mitochondria and other organelles are found (Lee and Atkinson 1976; Rosenbluth 1965a). The muscle cells are closely associated with hypodermis and connected to the cuticle by fibers passing from the contractile part of the cell (Rosenbluth 1965a; del Castillo et al. 1989). The nematodes do not possess circular body muscles which limits their movement pattern. They exhibit a characteristic waveform motion accomplished by contractions and relaxation of dorsal or ventral musculature in apposition with the rigid cuticular layer that limits the motion (Harris and Crofton 1957a; Gardner 2013; Stretton 1976; Weisblat and Russell 1976). The musculature of the nematode, *Ascaris* spp., is discussed in detail in the next section.

2.3 Nematode neuromuscular system

The neuromuscular system of the nematodes differs from other animals and is a good example of the multiple specialization in metazoan cells. Here we discuss the neuromuscular system of the *Ascaris* spp. The structure of helminth somatic muscle cells is unique as is the peculiar spatial relations of these cells with the nervous system. The nerves of these worms do not branch out to the muscle, but instead, an innervation process is extended from the non-contractile part of the muscle cells to terminate on one of the longitudinal nerves (Rosenbluth 1965a, 1963; Stretton 1976; del Castillo et al. 1989; Schneider 1866). Additionally, the neuromuscular system of nematodes, including *Ascaris* spp., have a fixed number of cells and cell divisions. Another characteristic

feature that makes nematodes different is the small and constant number of identifiable neurons in each worm species (del Castillo et al. 1989).

2.3.1 Nematode muscular system

The muscle cells in *Ascaris* are longitudinally directed and circumferentially arranged beneath the hypodermis. The somatic musculature is formed by 5x 10^4 number of mononucleated cells, separated into the dorsal and ventral fields by the two lateral hypodermal chords (Stretton 1976). Each muscle cell is geometrically complex and consists of three structurally distinct parts (Figure 2.4); a ribbon-like contractile part situated most peripherally, the more centrally located balloon-like 200 μm perinuclear part, known as the bag or belly, and lastly the slender, elongated innervation process, or arm, that extends from the belly and divides into several finger like projections that synapse on the nerve fibers (Rosenbluth 1965a). The contractile part of the muscle cells, also known as the spindle region, is anchored to the hypodermis. It is composed of obliquely striated (~2mm in length) myofibers that contains interdigitating arrays of thick and thin myofilaments (Rosenbluth 1963, 1965a). The muscle bag floats freely facing the pseudocoelom and contains the nucleus, submembrane mitochondria, particulate glycogen and other organelles (Rosenbluth 1965b). The non-myofibrillar muscle arms project from the base of the bag, cross the muscle field transversely to reach over the nerve cord surface, and eventually divide into thin, terminal projections or fingers just before receiving innervation from the nerve cord. Most muscle cells have a mean number of 2.7 arms (Stretton 1976). The fingers of the muscle arm intertwine with the fingers of adjacent arms, forming a ribbon-shaped plexus. This plexus extends longitudinally in close proximity to the nerve cords and constitutes an electrically coupled cap of

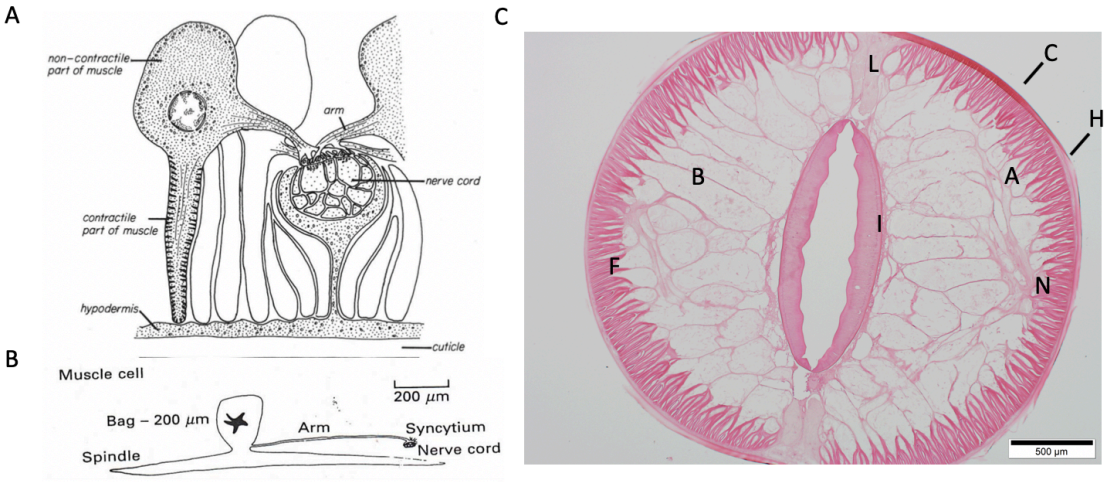

Figure 2.4 A. Transverse section of *Ascaris* muscle cells and myoneural junctions. The arm of the innervation process subdivides as it approaches the nerve cord (Lee and Atkinson 1976). B. *Ascaris* muscle cell showing the muscle bag, spindle, arm, syncytium and nerve cord (Martin et al. 1991). C. Transverse section of anterior section of *Ascaris* (The image was kindly provided by Mark Mchugh, Department of Biomedical Sciences, Iowa State University). The worm is surrounded by cuticle (C), beneath which lies the hypodermis (H). The intestine (I) lies between the two lateral lines (L). The somatic muscle fibers (F) are seen as radial structures subjacent to the hypodermis and are continuous with the balloon-like muscle bellies (B). The muscle arms (A) can be seen converging on a nerve cord (N).

interlocking processes, forming a functional syncytium (Rosenbluth 1965a; DeBell 1963). The syncytium and nerve cord fibers form the neuromuscular junctions in *Ascaris*.

2.3.2 Nematode nervous system

The nervous system of the nematodes is remarkably consistent. *Ascaris* spp. contains only about 250 neurons. It is composed of a nerve ring (or circumpharyngeal commissure) and associated ganglia in the head region, the major dorsal and ventral nerve cords and the smaller subdorsal and subventral cords, and another, smaller set of ganglia in the posterior region (Figure 2.5 A&B). The dorsal and ventral nerve cords pass caudally along the length of the body (Stretton et al. 1978). The motor nervous system is the region that is sufficient to exhibit locomotor behavior when the anterior and posterior 5mm of the worm is removed (Stretton et al. 1992; Stretton et al. 1978). The neurons of

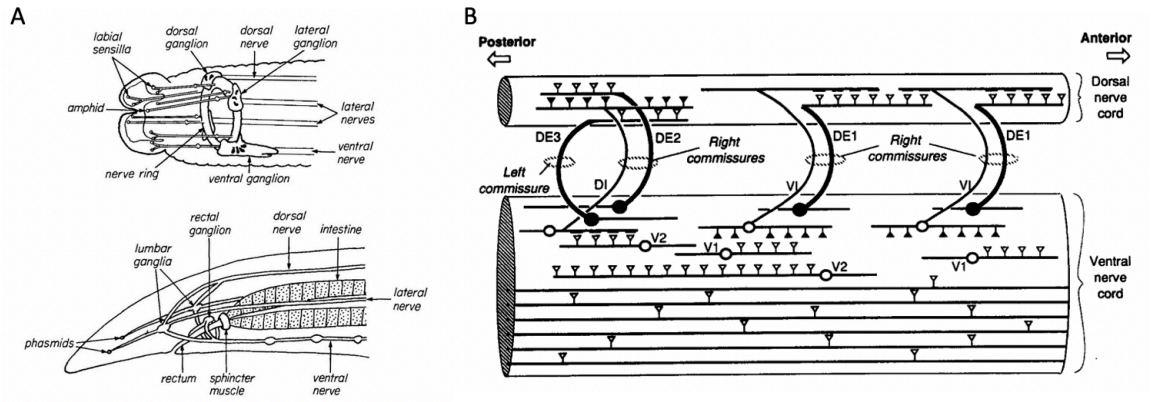

Figure 2.5 A. The nervous system of the head and the tail region of *Ascaris* female (Lee and Atkinson 1976). B. Organization of the motor nervous system in one segment of *Ascaris*. Each segment contains eleven motoneurons and six non-segmental interneurons. The seven morphological types of motoneurons are indicated as DI: dorsal inhibitory, VI: ventral inhibitory, DE1: dorsal excitatory 1, DE2: dorsal excitatory 2, DE3: dorsal excitatory 3, V1 and V2: ventral excitatory (Martin 1993).

the motor nervous system are concentrated in the ventral and dorsal nerve cords and classified into three categories: motor neurons, large interneurons and small interneurons. The motor neurons make synapses with muscle cells either in the dorsal nerve cord or the ventral nerve cord. The large interneurons make synapses onto certain motor neurons, while the small interneurons synapse onto each other or onto large interneurons. The cell bodies of the motor neurons are located in the ventral nerve cord, and they are linked to the dorsal nerve cord by single or paired lateral processes known as commissures. These commissures repeat in a pattern down the length of the body and each repeat, also called the segment, consists of three paired right-hand commissures and one left-hand commissure. The motor neurons are geometrically simple and have been classified into seven distinct types based on the morphological distribution of axons and dendrites. Each type of motor neuron is arranged serially along the length of the worm in five segments; each segment contains 11 copies of the cell (Figure 2.5 C). Out of the seven types, DI, DE2 and DE3 type motor neurons occur only once in each segment, while, the other four

types namely, DE1, VI, V1 and V2 occur twice. V1 and V2 are confined to the ventral nerve cord and the rest have processes in both the dorsal and ventral nerve cords (Stretton et al. 1992; Stretton et al. 1978; Martin 1993; Martin et al. 1991).

2.4 Nicotinic acetylcholine receptors (nAChRs)

Nicotinic acetylcholine receptors are pentameric membrane-spanning ligand-gated ion channels (LGICs). nAChRs mediate excitatory neurotransmission in both vertebrates and invertebrates through the finely tuned spatial and temporal regulation of ion flow across cell membranes. The discovery of the nAChRs played a key role in the advancement of pharmacology. These were the first membrane receptors of a neurotransmitter and ion channel to be characterized as a protein (Changeux 2012). The nicotinic actions were first encountered by Europeans when Columbus's crew sampled tobacco in 1492. The plant was named *Nicotiana* after Jean Nicot, the French ambassador to Portugal, who introduced it and later the active alkaloid of tobacco was named nicotine (Charlton 2004). The concept of a pharmacological receptor was discovered as a result of investigation to localize the physiological action of tobacco and curare. It was Claude Bernard, who in 1857, deduced that curare acted at the junction between motor nerve and muscle (Cousin 2013; Martindale and Lester 2014; Bennett 2000). Alfred Vulpian repeated Bernard's experiments in the 1860's and articulated that curare caused disruption of communication between nerve and muscle (Monassier 2017; Bennett 2000; Martindale and Lester 2014). However, his conclusions did not specify structure through which curare exerted its effects. In 1885, Paul Ehlrich suggested that toxins chemically interacted with the specific side-chains on the cell surface (Bosch and Rosich 2008).

Although, Ehlrich is credited with the term “chemoreceptors”, it was John Newport Langley, who in 1905 proposed that muscle contraction was mediated by a "receptive substance", which combined with nicotine and curare (Langley 1901, 1905; Bennett 2000). These “receptive substances” are presently designated as receptors. Langley concluded that the receptors could receive stimulus from the nerve and transmit it to the effector cell. David Nachmansohn (1955) postulated that receptors are proteins and it was not until 1970 that the first neurotransmitter nAChR (from electric ray, *Torpedo californica*) was identified, purified and biochemically characterized (Miledi and Potter 1971; Changeux, Kasai, and Lee 1970). In the 1980s, the nAChR family and an extended family of homologous pentameric receptors was identified using molecular biology techniques (Le Novère and Changeux 1995; Patrick et al. 1983). With the advent of more refined scientific techniques since then, various nAChR subunits have been identified and cloned. The agonist binding sites and the channel-lining residues have also been mapped in the subunit sequences. Using electron microscopy, the shape of the receptor complex and the arrangement of its subunits have been deciphered (Corringer, Novère, and Changeux 2000; Sumikawa et al. 1982; Karlin 2002; Le Novère, Corringer, and Changeux 1999; Miyazawa et al. 1999; Unwin 2005; Kao et al. 1984). High resolution structure (4.0 Å and 4.6 Å) of the marine ray *Torpedo* nAChR has been invaluable and provided useful insights into the structure of cys-loop LGICs (Miyazawa et al. 1999; Unwin 2005). The discovery of the acetylcholine binding protein crystal structure from *Lymnea stagnalis*, has offered detailed three-dimensional information about the relative positioning of the principal and complementary part of the ligand-binding site (Brejc et al. 2001). Thus, nAChRs have been paramount in the discovery of

neurotransmitter receptors and arguably the most thoroughly characterized of all the ligand-gated ion channels.

The nicotinic acetylcholine receptors are the prototype of the cys-loop family of ligand-gated ion channels that also includes ionotropic receptors for 5-hydroxytryptamine (5-HT), γ-aminobutyric acid (GABA), glycine, histamine and glutamate (Thompson, Lester, and Lummis 2010b). nAChRs are cell-surface polytopic complexes composed of five co-assembled subunit proteins, arranged to form a rosette with a central non-selective cation conducting pore (Changeux 2012). Each receptor has an extracellular ligand-binding domain, cys-loop (two cysteine residues separated by 13 amino acids), six loops (A-F), four transmembrane domains (TM1 – TM4) that allows ions to pass across the membrane, and an intracellular cytoplasmic domain that plays a role in channel conductance and receptor modulation (Figure 2.6). These receptors are assembled from a diverse collection of subunits (Sine and Engel 2006; Corringer, Novère, and Changeux 2000). The subunits include α-subunits which differ from non-α subunits by having "vicinal" (adjacent) cysteines residues on the extracellular loop-C. These cysteines are believed to be essential in ligand binding and thus, the binding of the ligands occurs at the interface of an α-subunit and an adjacent subunit (Kao et al. 1984; Arias 1997). The nAChRs can be homomeric (containing five copies of a single α-subunit) or heteromeric (multiple subunits with at least 2 α) (Corringer, Novère, and Changeux 2000; Karlin 2002). nAChRs are expressed in the nervous system, at the neuromuscular junction (Changeux and Edelstein 2006) as well as in the non-neuronal cells such as epithelial cells of bronchi, endothelial cells of the arteries, macrophages, and

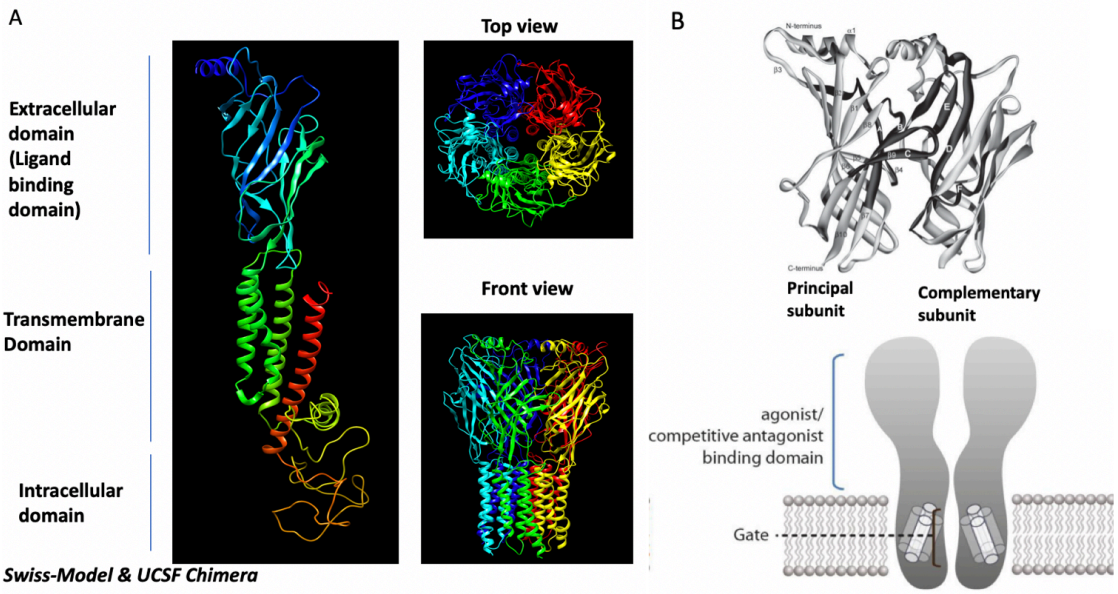

Figure 2.6 Structure of nicotinic acetylcholine receptors. A. Three-dimensional protein structure (ribbon structure) of individual subunit showing the extracellular, transmembrane and intracellular domain. Each nAChR consists of five subunits arranged around a cationic channel pore. Representation of the assembled receptor is shown viewed from the top and front. All the subunits are shaded in a different color differently to indicate clear boundaries. B. The ligand binding site is present at the interface of two adjacent subunits. The binding loops and β-sheets are shown in the image. The ligand binds between the loops A, B and C from principal subunit and loops D, E and F from the complementary subunit. This leads to opening of the cation channel that results in prolonged activation of the excitatory nicotinic acetylcholine receptors on nematode muscles leading to spastic paralysis and expulsion of worms (Thompson, Lester, and Lummis 2010b; Nasiripourdori et al. 2011).

keratinocytes of the skin; suggesting a broader functional spectrum (Macklin et al. 1998; Bruggmann et al. 2002; Wang et al. 2003; Wessler et al. 2003). The nAChRs are widely distributed throughout the animal kingdom and a large number of nAChR subunits have been identified in both vertebrates and invertebrates (Le Novère and Changeux 1995). These subunits co-assemble in various combinations, each arrangement conferring different functional and pharmacological properties. Even the precise order and stoichiometry of subunits in the pentameric channel produces different response profile

(Le Novère, Corringer, and Changeux 2002; Buxton et al. 2014). Thus, the structural and functional diversity within this receptor family has made them potential therapeutic targets for a wide variety of medical conditions (Millar and Gotti 2009; Jones and Sattelle 2004). There is kindled interest in development of subtype-selective ligands for management of various neurodegenerative disorders such as Alzheimer's disease, Parkinson's syndrome and schizophrenia (Leonard and Bertrand 2001). In invertebrates, nAChRs are also of considerable interest as they are targets for leading insecticides (Millar and Denholm 2007) as well as the antiparasitic drugs levamisole, pyrantel and monepantel (Abongwa, Martin, and Robertson 2017; Martin et al. 1991). The nAChRs of vertebrates and invertebrates share many structural and functional properties. But there are important differences in their physiological roles and pharmacological properties (Millar and Denholm 2007).

2.4.1 Vertebrate nAChRs

Vertebrates possess 17 nAChR subunits (α1–α10, β1–β4, γ, δ and ε) which are classified into two subtypes, the 'muscle' and the 'neuronal' nAChRs (Le Novère and Changeux 1995; Pedersen, Bergqvist, and Larhammar 2019). Of the 17 subunits identified so far, the α8 subunit is expressed in avian species but not in mammals. The muscular ion channels are heteromeric and have a subunit composition of $(\alpha1)_2$ $(\beta)_1\varepsilon\delta$ in adults or $(\alpha1)_2$ $(\beta)_1\gamma\delta$ in the fetus (Missias et al. 1996). The acetylcholine binding sites are located at the α-δ and α-ε interfaces in the muscle type nAChRs. The adjacent subunit (δ or γ/ε) also contribute to the agonist binding site, although occupancy of both binding sites is required for effective opening of the channel (Nayak and Auerbach 2013; Karlin 2002). The neuronal nAChRs are composed of 9 alpha subunits (α2 – α10) and 3 beta

subunits (β2 – β4) (Galzi and Changeux 1995; Dani 2015; Zoli, Pistillo, and Gotti 2015). These receptors can be either homopentamers or heteropentamers consisting of two or three types of subunits such as (α4)3(β2)2 or (α4)2(β2)3. This enables greater diversity in subunit composition, consequently different biophysical and pharmacological properties (Gotti, Zoli, and Clementi 2006; Wu, Liu, et al. 2016; Zoli, Pistillo, and Gotti 2015; Li et al. 2016). Only α-7, α-8, and α-9 subunits reconstitute functional homomeric nAChRs in *Xenopus* oocytes (Couturier et al. 1990; Elgoyhen et al. 1994; Gerzanich, Anand, and Lindstrom 1994). The two agonist binding sites in neuronal receptors (α-β and α-α interfaces) have different affinities. The α5 and β3 subunits behave as the accessory subunits as they do not directly participate in formation of the binding site (Kuryatov, Onksen, and Lindstrom 2008). The vertebrate nAChR subunits have been further divided into four subfamilies based on sequence similarities and resemblance of gene structures (position of the introns in the coding sequence): subfamily I, epithelial α9; subfamily II, neuronal α7, 8; subfamily III, neuronal α2–6 and β2–4; and subfamily IV, muscle α1, β1, γ, δ, and ε (Le Novère, Corringer, and Changeux 2002; Le Novère and Changeux 1995). The neuronal nAChRs modulate a variety of neuronal functions including learning, memory, arousal, cerebral blood flow and metabolism (Cordero-Erausquin et al. 2000; Levin and Simon 1998). Mutations in nAChR subtypes have been linked to myasthenia gravis, congenital myasthenia syndrome and certain epilepsies (Itier and Bertrand 2002; Steinlein et al. 1995; Lindstrom 2003). Neuronal nAChRs have also been implicated in several neurological disorders including attention deficit disorders, Alzheimer's disease, epilepsy, Parkinson's disease, schizophrenia and Tourette's syndrome (Clementi, Fornasari, and Gotti 2000; Paterson and Nordberg 2000; Weiland,

Bertrand, and Leonard 2000; Picciotto and Zoli 2002). Additionally, nicotine dependence observed with consumption of tobacco and smoking is due to nicotine which acts on nAChRs (Greenbaum and Lerer 2009; Mansvelder and McGehee 2002; Dani, Ji, and Zhou 2001). Thus, these cy-loop ion channels are the focus of intense neuropharmacological research as therapeutic targets for various neurodegenerative diseases, as well targets to treat alcohol and nicotine dependence.

2.4.2 *Caenorhabditis elegans* nAChRs

Although *C. elegans* (Clade V) is one of the simplest organisms to employ acetylcholine as a neurotransmitter. It possesses the largest and most diverse nAChR gene family described to date. The nAChRs are expressed in nerve and muscle cells as well as at both synaptic and non-synaptic sites (Jones and Sattelle 2004; Holden-Dye et al. 2013). There are 32 nAChR subunits in *C. elegans* which are divided into five "core" groups based on sequence homology (Jones and Sattelle 2004; Bargmann 1998; Holden-Dye et al. 2013). The groups are UNC-29 group, UNC-38 group, ACR-8 group, ACR-16 group and DEG-3 group (Figure 2.7). Each group is named after the first subunit to be characterized and majority of the subunit genes are denoted *acr* for ***a***cetyl***c***holine ***r***eceptor (Mongan et al. 1998; Jones et al. 2007). Among the 32 nAChR subunits, 22 have been classified as α. The members of ACR-16 group are highly homologous to vertebrate α7–10 nAChR subunits (Mongan et al. 1998; Ballivet et al. 1996). The DEG-3 andACR-8 are unique to nematodes while UNC-38 group members most closely resemble insect nAChR subunits (Mongan et al. 2002). The UNC-29-like subunits show similarity to AChR-like Drosophila protein (ARD; non-α subunit) of *Drosophila* and vertebrate muscle non-α subunits (Jones and Sattelle 2004). All the

receptor subunits show characteristics common to nAChRs. Although, several subunits possess unusual modifications that alter their pharmacological properties (Figure 2.8). For example, two members of the DEG-3 group, ACR-24 and ACR-5, contain FxCC in loop-C instead of the highly conserved YxCC motif, which decreases the affinity for acetylcholine (Galzi et al. 1991). In ACR-10, subunits of ACR-8 group and UNC-38 the YxCC motif is instead replaced by YxxCC (Mongan et al. 1998). The ACR-8 group members are the only subunits in which a basic residue (histidine), instead of the highly-conserved acidic residue, flanks the TM2 region. All these modifications and diversity in key motifs likely results in receptors with different biophysical and pharmacological properties (Jones and Sattelle 2004). *C. elegans* also possesses additional 26 subunits denoted as "orphan" subunits which are homologous to nAChRs but do not fall within the core groups. None of these subunits are α subunits and their function is unknown, except for CUP-4 and LGC-26 which are involved in endocytosis (Jones et al. 2007; Patton et al. 2005).

Different nAChR subunits can combine into four major types of nAChRs in *C. elegans*, viz, levamisole-sensitive, nicotine-sensitive, DEG-3/DES-2 and EAT-2 nAChRs (Figure 2.9). These receptor types are essential for a variety of physiological behaviors including: feeding, locomotion, reproduction and development. They vary in their expression patterns and pharmacological properties. DEG-3/DES-2 receptors are localized to non-synaptic regions and the sensory endings of chemosensory neurons (Treinin et al. 1998). Levamisole-sensitive and nicotine-sensitive receptors are expressed in body wall muscle (Richmond and Jorgensen 1999). EAT-2 nAChRs are expressed in

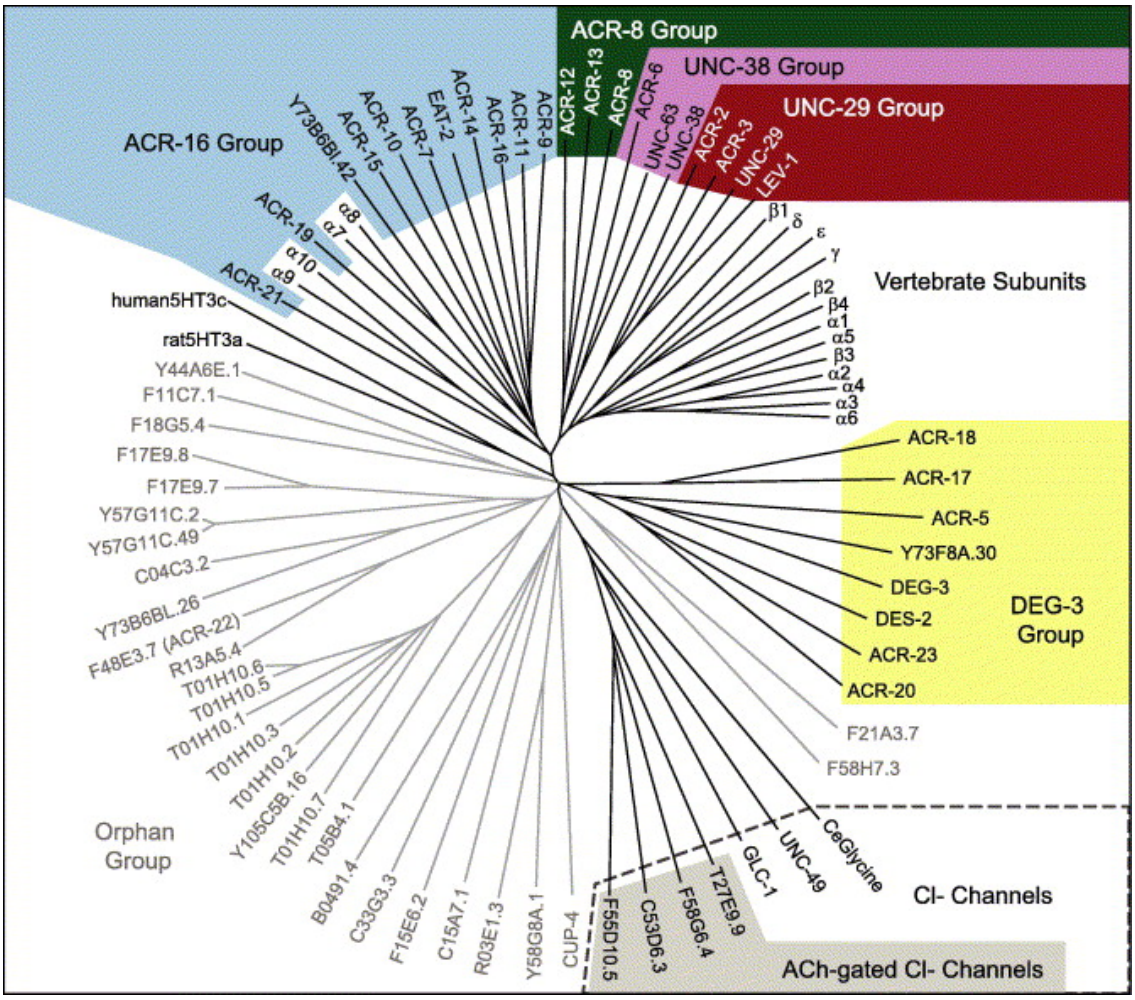

Figure 2.7 Tree showing the five core groups of nAChR subunits (indicated by the colored regions) and the orphan group (Brown et al. 2006).

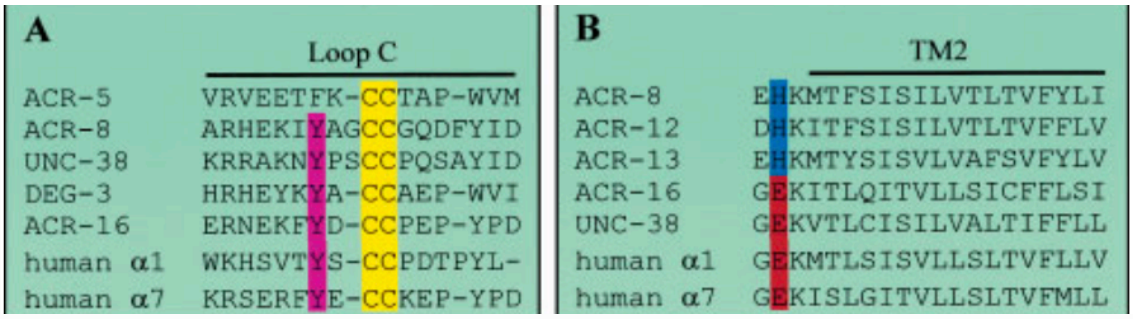

Figure 2.8 The unusual sequence features of the *C. elegans* nAChR subunits (Jones and Sattelle 2004). A. Alignment of Loop C from various nAChR subunits highlighting alterations in the YxCC motif is shown (Tyrosine residue highlighted in purple and the vicinal cysteines highlighted in yellow). B. Alignment of cytoplasmic domain of the ACR-8 nAChR subunit with various other subunits. The residue important for ion selectivity is shown either in blue (basic) or red (acidic).

the pharyngeal muscles along with a small transmembrane protein, EAT-18 (McKay et al. 2004).

Levamisole-sensitive (L-type) receptors are the most widely explored nAChRs in nematodes. They mediate excitatory neurotransmission at the neuromuscular junctions of nematodes and constitute a major target for anthelmintic treatments. Two non-α-subunits (LEV-1 and UNC-29) and three α-subunits (LEV-8, UNC-38, UNC-63) have been identified as important for levamisole sensitivity (Culetto et al. 2004; Jones and Sattelle 2004; Towers et al. 2005; Boulin et al. 2008; Fleming et al. 1997). Fleming et al. (1997) demonstrated that *lev-1*, *unc-29* and *unc-38* mediated levamisole resistance and reconstituted the receptor in *Xenopus* oocytes using the subunits. But the expression was low with small current responses to levamisole and the responses were inhibited by nicotinic antagonists, mecamylamine and neosurugatoxin. The low expression pointed to a possibility of involvement of additional subunit. *In vivo* recordings from *C. elegans* body wall muscles also confirmed the presence of receptors that were sensitive to levamisole and were completely blocked by d-tubocurarine (Richmond and Jorgensen 1999). The locomotion was impaired in UNC-29 and UNC-38 mutants in *C. elegans* (Richmond and Jorgensen 1999), confirming the role of the levamisole-sensitive nAChRs in locomotor behavior. Subsequently, additional genes, *unc-63* and *lev-8* (also called *acr-13),* encoding for nAChR subunits were found to be associated with levamisole resistance (Culetto et al. 2004; Towers et al. 2005). Further recombinant expression studies revealed the requirement of five nAChR subunits in addition to three ancillary proteins, RIC-3, UNC-50 and UNC-74, for successful *in vitro* expression of the heteromeric receptor (Boulin et al. 2008). The ancillary proteins, RIC-3, UNC-50 and UNC-74, are important

molecular components of the nAChRs. RIC-3 (***R***esistance to ***I***nhibitors of ***C***holinesterase) is an endoplasmic reticulum transmembrane protein. It acts as a chaperone to promote receptor folding, assembly or maturation (Millar 2008; Halevi et al. 2002). The genes, *unc-50* and *unc-74* (***unc***oordinated), were identified during mutational screening for levamisole resistance and mutants of these genes lack high-affinity levamisole binding (Lewis et al. 1980). UNC-50 is a transmembrane protein of the Golgi complex that prevents lysosomal targeted destruction of the subunits of levamisole sensitive nAChR during intracellular trafficking (Eimer et al. 2007). UNC-74 is a thioredoxin containing protein closely related to the human Thioredoxin Related Transmembrane protein, TMX3. It is required for trafficking the nAChR subunits to the synapses (Haugstetter, Blicher, and Ellgaard 2005). The heterologously expressed levamisole-sensitive nAChR was strongly inhibited by d-tubocurarine, methyllycaconitine and hexamethonium. Dihydro-β-erythroidine and α-bungarotoxin produced only modest blocking effects on the L-type nAChR (Boulin et al. 2008). *C. elegans* body wall muscles also express the ACR-8 subunit (Holden-Dye et al. 2013; Touroutine et al. 2005). It acts as a spare subunit for the levamisole sensitive nAChRs and may replace LEV-8 in its absence (Hernando et al. 2012).

Richmond and Jorgensen (1999) had identified a rapidly desensitizing nicotine and acetylcholine sensitive receptor, in addition to levamisole sensitive nAChRs, in *C. elegans* body wall muscles. *unc-29* mutants were responsive to nicotine and this response was completely blocked by dihydro-β-erythroidine. In wild type worms, the acetylcholine response was only about 50% blocked by dihydro- β-erythroidine. Mutants of *unc-29* and *unc-38* displayed impaired locomotion but were still responsive to acetylcholine,

suggesting an equal contribution of the nicotine-sensitive receptors. In *C. elegans*, the nicotine-sensitive nAChR (N-type nAChR) is a homopentamer formed by co-assembly of ACR-16 subunits (initially designated Ce21), which is a closely related homologue of the vertebrate α-7 subunit (Raymond, Mongan, and Sattelle 2000; Ballivet et al. 1996). N-type receptors only required RIC-3 only for successfully assembly into a functional ion channel. Levamisole did not act as an agonist on the nicotine-sensitive receptor, but rather antagonized the acetylcholine mediated responses. Dihydro-β-erythroidine and d-tubocurarine produced potent antagonistic effects on *C. elegans* ACR-16. The receptor is relatively insensitive to methyllycaconitine and α-bungarotoxin, both of which are potent antagonists of α-7 nAChRs. The heterologously expressed homomeric receptor desensitized rapidly following application of acetylcholine or nicotine. Nicotine is more potent agonist than acetylcholine of both vertebrate α-7 nAChRs and *C. elegans* ACR-16 receptors. However, it is a full agonist on α-7 cation channels but a partial agonist of the ACR-16 receptor (Couturier et al. 1990; Raymond, Mongan, and Sattelle 2000; Ballivet et al. 1996). Although, no behavioral phenotype is observed in *acr-16* mutants*, unc-63*:*acr-16* and *unc-29*:*acr-16* double mutants in *C. elegans e*xhibit locomotor defects which are more severe than single mutants of either *unc-63* or *acr-16* alone or *unc-29* single mutants (Touroutine et al. 2005; Li et al. 2014). This shows that ACR-16 in combination with UNC-63 and UNC-29, components of levamisole-sensitive nAChR, contributes to locomotor behavior in the worms.

The DEG-3 group of nicotinic acetylcholine receptor subunits is only found in nematodes (Brown et al. 2006). DEG-3 (***deg***eneration of certain neurons) and DES-2 (***de***generation ***s***uppressor), members of the DEG-3 group, are encoded by a pair of

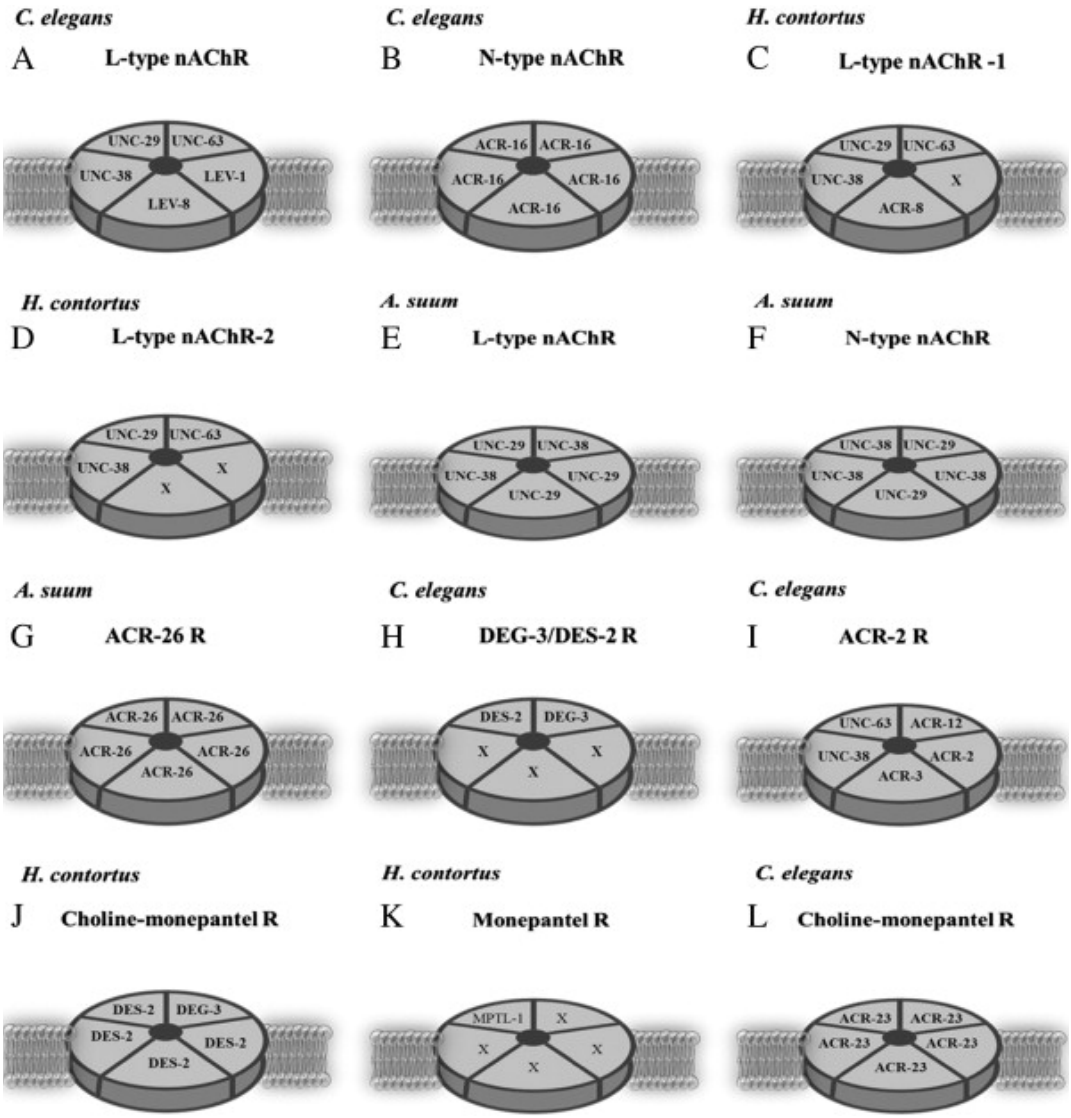

Figure 2.9 Proposed stoichiometry of nAChRs in *C. elegans* and parasitic nematodes. The figure shows possible arrangement and subunit combinations for the various nAChR types. This is based on *in vivo* recordings from body wall muscles and the studies on heterologously expressed nAChRs in *Xenopus* oocytes (Holden-Dye et al. 2013).

adjacent genes that form an operon that regulate the coordinated expression of the two subunits (Treinin et al. 1998). Neither of the subunits form homomeric channels in *Xenopus* oocytes but when co-expressed, generate a functional heteromeric receptor that has strikingly higher affinity for choline than acetylcholine. DEG-3 and DES-2 are localized in sensory endings of chemosensory neurons (Yassin et al. 2001). DEG-3

mutants display defective chemotaxis towards choline. Thus, DEG-3 and DES-2 possibly have an unusual role of regulating chemotaxis behavior (Yassin et al. 2001; Jones and Sattelle 2004). An aminoacetonitrile derivative, monepantel (AAD-1566), preferentially activates nAChR subunits of DEG-3 group, mainly ACR-23, leading to spastic paralysis in the worms (Kaminsky and Rufener 2012; Kaminsky et al. 2008). ACR-23 nAChRs are expressed in the *C. elegans* body wall muscles and unidentified neurons in head and tail (Kaminsky and Rufener 2012). ACR-23 homomeric receptors expressed in *Xenopus* oocytes responded to monepantel, choline, nicotine, and acetylcholine, with monepantel strongly potentiating choline mediated responses (Rufener et al. 2013). ACR-20, another member of the DEG-3 group, co-assembles into homomeric nAChRs when heterologously expressed in *Xenopus* oocytes. At low concentrations (< 1 nM), monepantel produced positive allosteric modulatory effects on the *C. elegans* ACR-20 nAChR, while at high concentrations (> 0.1 μM) the aminoacetonitrile derivative acted as a direct agonist of the receptor.

C. elegans pharyngeal muscles express a non-alpha nAChR subunit, EAT-2, which is required for regulation of pharyngeal pumping. It is localized postsynaptically on pharyngeal muscles, between pm4 and pm5, which is also the site of the MC motor neuron and its synapse. The nAChR subunit interacts with a small protein, EAT-18, which is also expressed in the pharyngeal muscles (McKay et al. 2004; Avery 1993a; Raizen, Lee, and Avery 1995). The pharyngeal nAChRs are not a well-exploited target for anthelmintic drug development.

2.4.3 Parasitic nematode nAChRs

The nematode nAChRs on muscle are the target of the cholinergic anthelmintics including levamisole, pyrantel, morantel and oxantel. These compounds activate these ligand-gated cation channels, leading to prolonged muscle contraction and spastic paralysis of the parasite (Aceves, Erlij, and Martínez-Marañón 1970; Aubry et al. 1970; Martin et al. 1991; Dale and Martin 1995). The nAChR of parasitic nematodes are different from the *C. elegans* receptors in their subunit composition and resulting biophysical and pharmacological properties. The parasitic nematode *A. suum* (Clade III) has been extensively used in the characterization studies of the native body wall muscle nAChRs, largely due to their large muscle cells. Pennington and Martin (1990) for the first time described acetylcholine-activated single channel currents in the *Ascaris suum* muscles. They used inside-out patch clamp recordings and revealed the presence of at least two acetylcholine-activated channel types. Using single channel patch-clamp recording techniques, Robertson and Martin (1993), examined the levamisole-activated channels in *A. suum* muscle vesicles. Low levamisole concentrations (1–10 μM) caused channel opening, while high levamisole concentrations (30 and 90 μM) caused an additional flickering channel block. The channel conductance was in the range of 18–50 pS. Pyrantel and oxantel were also shown to act as agonists and open channel blockers of the nAChRs by using patch clamp single-channel recordings from muscle vesicles of somatic muscle cells of *Ascaris suum* (Robertson et al. 1994; Dale and Martin 1995). Robertson et al. (2002) and colleagues used paraherquamide and its semisynthetic derivative, 2-deoxyparaherquamide or derquantel, to distinguish cholinergic receptor subtypes in *A. suum* muscle. Based on the antagonist effects produced by these

compounds on muscle contractions elicited by nicotine, levamisole, pyrantel and bephenium, three subtypes of muscle nAChRs were identified. These nAChR types are the L-type preferentially activated by levamisole, the N-type preferentially activated by nicotine and the B-type preferentially activated by bephenium. The channel conductance and mean open times for these nAChR types have been resolved at the single-channel level by using various agonists and antagonists (Qian, Martin, and Robertson 2006). The single channel conductance and mean open times are respectively, 24 pS and 0.6 ms for the N-type, 35 pS and 0.9 ms for the L-type, and 45 pS and 1.3 ms for the B-type (Figure 2.10A). The N-subtype was activated by oxantel, methyridine; L-subtype by pyrantel and competitively antagonized by paraherquamide; B-subtype was competitively antagonized by paraherquamide and derquantel. Unlike *C. elegans*, the three types of nAChRs in *A. suum* show subtle differences in agonist pharmacology. For example, levamisole not only activates L-type channels but can also stimulate the opening of N- and B-type channels to a lesser extent. Similar, bephenium is not just selective for B-type channels but also activates some L-type but not N-type channels. A high concentration of an nAChR agonist, such as levamisole can lead to activation of all three nAChR types in *A. suum* (Robertson et al. 2002; Qian, Martin, and Robertson 2006; Martin et al. 2004).

In *Oesophagostomum dentatum*, a clade V nematode, an additional fourth levamisole-activated subtype with a single-channel conductance of 40 pS has been revealed (Figure 2.10B) (Robertson, Bjorn, and Martin 1999). The G35 channel, present in levamisole-sensitive (SENS) parasites, was not present in levamisole-resistant (LEVR) parasites. Thus, like *A. suum*, the G35 channel is the L-type nAChR in *O. dentatum*. In pyrantel-resistant *O. dentatum*, even though four conductance states of the nicotinic

receptors were present there was reduction in the percentage of active patches and probability of channel opening (P_o) in anthelmintic resistant worms (Robertson, Bjørn, and Martin 2000). Based on single-channel recording, the parasitic nematodes display a high level of complexity and heterogeneity in nAChRs.

Buxton et al. (2014) characterized the effects of varying subunit composition and stoichiometry of four *O. dentatum* nAChR subunits on the pharmacological profile of the cation channel expressed in *Xenopus* oocytes (Figure 2.10C). The authors identified four distinguishable nAChR subtypes based on agonist sensitivities, the pyrantel (Pyr)-nAChR composed of UNC-29 and UNC-63 subunits, the pyrantel/tribendimidine (Pyr/Tbd)-nAChR composed of UNC-29, UNC-63 and UNC-38 subunits, the acetylcholine (ACh)-nAChR composed of UNC-29, UNC-63 and ACR-8 subunits, and the levamisole (Lev)-nAChR composed of UNC-29, UNC-63, UNC-38 and ACR-8 subunits. The LEV-8 subunit has not been detected in any nematode parasite, however, a related subunit, ACR-8, can be used to reconstitute the receptors. It is plausible the L- and N-type nicotinic receptors in the pathogenic clade III nematodes may be different from that found in *C. elegans*. The single channel conductance and mean open times observed for levamisole-sensitive channels (35 pS and 2.4 ms, respectively) were the same as previously identified *in vivo*. Even varying the subunit stoichiometry of the pyrantel-nAChR from 1:5 ratio of UNC-29 and UNC-63 subunits to 5:1 produced change in agonist pharmacology. The responses to pyrantel and tribendimidine were significantly increased with 1:5 stoichiometry, and when the ratio was reversed, the nicotine and levamisole responses became significantly bigger than the acetylcholine response.

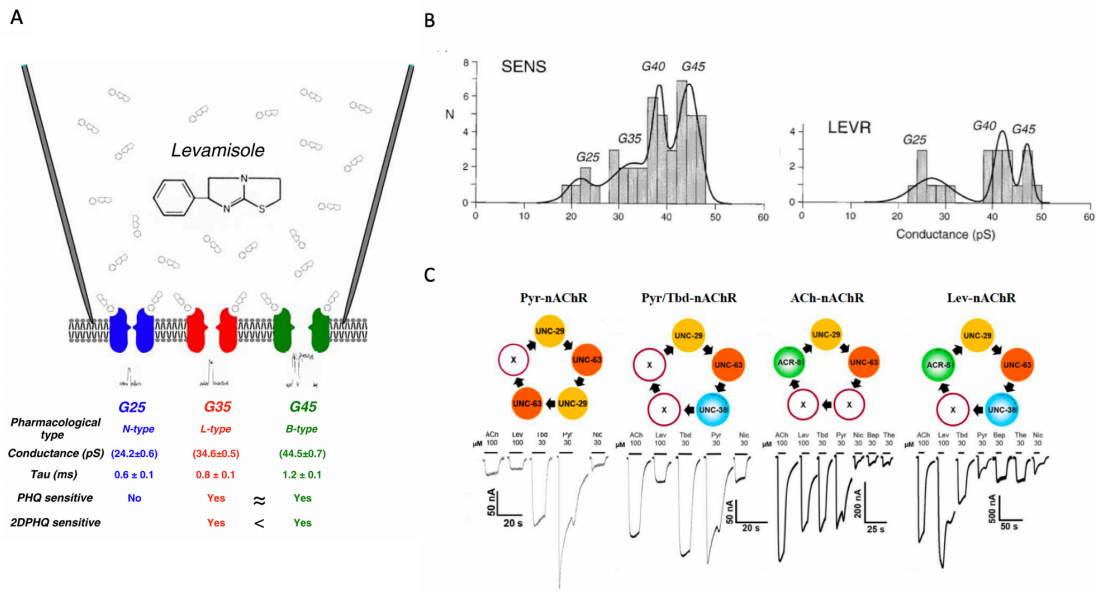

Figure 2.10 A. Summary diagram of nAChR subtypes on *Ascaris suum* somatic muscle cell membrane. The single channel and pharmacological properties of each subtype are indicated. N-subtype prefers nicotine, L-subtype prefers levamisole and B-subtype prefers bephenium. The subtypes show different sensitivities to the competitive antagonists paraherquamide (PHQ) and 2-desoxoparaherquamide (2DPHQ) (Qian, Martin, and Robertson 2006). B. Frequency histograms of single channel conductance for SENS and LEVR parasites (Robertson, Bjorn, and Martin 1999). C. Summary of the four *O. dentatum* nAChR subtypes based on different subunit composition reconstituted in *Xenopus laevis* oocytes. The pyrantel (Pyr)-nAChR is composed of UNC-29 and UNC-63 subunits, the pyrantel/tribendimidine (Pyr/Tbd)- nAChR is composed of UNC-29, UNC-63 and UNC-38 subunits, the acetylcholine (ACh)- nAChR is composed of UNC-29, UNC-63 and ACR-8 subunits, and the levamisole (Lev)- nAChR is composed of UNC-29, UNC-63, UNC-38 and ACR-8 subunits (Buxton et al. 2014).

The heteromeric levamisole-sensitive nAChR can be recapitulated by expressing *A. suum* UNC-38 and UNC-29 in *Xenopus* oocytes (Williamson et al. 2009). Both the subunits are colocalized in the body wall muscles of the nematode. The relative expression levels of the two subunits, altered the sensitivity of the agonists for the receptor. The oocytes injected with 5:1 and 1:5 ratio of UNC-38:UNC-29 produced more robust current responses as well a striking differences in agonist pharmacology as compared to ratios of 10:1 and 1:10. When the UNC-38:UNC-29 ratio was 5:1, nicotine acted as a full agonist and levamisole acted as a partial agonist. Levamisole behaved as a

full agonist and nicotine a partial agonist when the ratio was reversed to 1:5. With the 5:1 ratio, oxantel produced agonistic effect but not pyrantel, while with the 1:5 ratio, oocytes responded to pyrantel but not oxantel.

Haemonchus contortus, a clade V nematode, possesses genes encoding UNC-38, UNC-29, UNC-63 and ACR-8 that assemble into the heteromeric L-type nAChRs. No orthologue of the *C. elegans* LEV-8 L-AChR subunit was identified in *H. contorus* (Neveu et al. 2010). ACR-8 nAChR subunit is closely relate to the *lev-8* gene and has been implicated in the molecular mechanisms leading to levamisole resistance (Boulin et al. 2011). Blanchard et al. (2018) have demonstrated the critical role of the ACR-8 subunit in the levamisole sensitivity of parasitic nematodes using heterologous expression systems and gene silencing techniques. By varying the subunit composition, two types of L-AChRs, *Hco*-L-AChR1 and *Hco*-L-AChR2, can be functionally reconstituted *in vitro* (Boulin et al. 2011; Charvet et al. 2012). The co-expression of UNC-38, UNC-29, UNC-63 and ACR-8 constitutes the *Hco*-L-AChR1. This L-nAChR subtype was more sensitive to levamisole than acetylcholine and weakly sensitive to pyrantel, bephenium and nicotine. The *Hco*-L-AChR2 requires only three subunits, UNC-38, UNC-29 and UNC-63. This subtype was more sensitive to pyrantel and acetylcholine than levamisole and insensitive to bephenium. Gene duplication of the *unc-29* L-AChR subunit gene has led to the expression of four copies of the *unc-29* gene (*Hco*-UNC-29.1, *Hco*-UNC-29.2, *Hco*-UNC-29.3 and *Hco*-UNC-29.4) in *H. contortus* (Duguet et al. 2016; Neveu et al. 2010). This adds to the complexity and diversity of the nAChRs in the parasitic nematodes. Three functional receptor types with different pharmacological profiles were observed when each of the four UNC-29 copies were substituted in the Hco-L-AChR1

receptor. *Hco*-UNC-29.2 failed to reconstitute a functional receptor. When UNC-29.1 was used, the L-type nAChR (L-AChR1.1) was more responsive to levamisole than acetylcholine and mecamylamine was a potent antagonist of L-AChR1.1. When UNC-29.3 was used instead of UNC-29.1 (L-AChR1.3), the acetylcholine response was greater than the levamisole and mecamylamine was a less potent antagonist. With UNC-29.4 (L-AChR1.4), levamisole and acetylcholine responses were about equal and mecamylamine was more potent.

Abongwa, Martin, and Robertson (2017) reconstituted a homomeric nAChR, ACR-16, from *A. suum* in the *Xenopus* oocytes. The ACR-16 nAChR is most closely related to α-7 vertebrate receptors. Strikingly, the *Asu*-ACR-16 was expressed not only in the somatic muscle but also in the pharynx, ovijector, head and intestine implying the nAChR may have additional tissue-related functions in the worm. Similiar to *C. elegans*, the *A. suum* channel was most sensitive to nicotine but insensitive to levamisole and pyrantel. Unlike the *C. elegans* ACR-16 homologue, the *A. suum* channel was highly sensitive to mecamylamine but moderately sensitive to hexamethonium and DhβE (Briggs, McKenna, and Piattina-kaplan 1995). ACR-16 homologues from both the species were nearly insensitive to α-bungarotoxin, but highly sensitive to d-tubocurarine (Zhao et al. 2003). In contrast, α-7 vertebrate receptors are highly sensitive to α-bungarotoxin. The positive allosteric modulators of α-7 (ivermectin, genistein and PNU120596) produced inhibitory effects on *Asu*-ACR-16 (Ballivet et al. 1996). *Asu*-ACR-16 has 50 times smaller relative calcium permeability ratio than that of the α7 receptor (Seguela et al. 1993). In *Brugia malayi*, a clade V worm, knockdown of *acr-16: acr-26* had no effect on motility (Verma et al. 2017) suggesting a different physiological

function of the ACR-16 homologue in the filarial worm. ACR-16 homologue distribution is more widespread in *A. suum*. It is plausible that the ACR-16 nAChRs not only regulates neurotransmission in *A. suum* but also serves other tissue-related functions including reproduction and digestion.

2.4.4 nAChR auxiliary subunits

Biogenesis of multimeric membrane proteins, including nAChRs, is a complex and tightly regulated multistep process. It involves subunit folding, assembly of subunits into pentamers, trafficking to the cell surface, and finally, targeting to specific subcellular compartments. As mentioned before, ancillary or chaperone proteins, including RIC-3, UNC-50 and UNC-74, ensure proper protein folding, subunit assembly, and trafficking of the pentameric complexes to the cell surface in a subtype-dependent manner (Halevi et al. 2002; Haugstetter, Blicher, and Ellgaard 2005; Eimer et al. 2007). Another unique group of proteins, known as the auxiliary proteins, existing within the biological matrix of multimeric proteins has been identified for ionotropic receptors. These proteins directly associate with the ion channels and affect various aspects of biological functions, including forward trafficking, surface delivery and gating kinetics. The presence of auxiliary proteins has helped in addressing the discrepancies observed when comparing recombinant and native biophysical properties and pharmacological sensitivities of the ion channels (Isom, De Jongh, and Catterall 1994; Adelman 1995). There are four criteria which define the framework for classifying auxiliary proteins (Yan and Tomita 2012):
i) An auxiliary protein should not be a pore-forming subunit and should not form functional ion-conducting pores of its own.

ii) An auxiliary protein should interact with the pore-forming protein in a direct, stable and specific manner.

iii) The natural interaction of auxiliary subunit with the pore-forming subunit must result in a functional consequence. Specifically, the auxiliary protein must modulate the channel properties and/or assist in the trafficking of the receptor in heterologous cell systems.

iv) An auxiliary subunit must have demonstrable biological impact on certain aspects of native channel function *in vivo*.

The ancillary and auxiliary proteins have different roles in controlling channel functions *in vivo*. An ancillary or a chaperone assists with folding, assembly and trafficking of a protein complex. The interaction of the ancillary protein with the channel subunits is transient and not stable. Therefore, these proteins do not meet the criteria of an auxiliary subunit.

The auxiliary proteins were first identified for voltage-gated ion channels in the early 1990s (O'Malley and Isom 2015; Adelman 1995; Gurnett and Campbell 1996; Isom, De Jongh, and Catterall 1994). However, it was not until 2000s that the first auxiliary protein, Stargazin, a small transmembrane AMPA receptor regulatory proteins (TARPs), was identified for the glutamate-gated AMPA receptor (Chen et al. 2000; Osten and Stern-Bach 2006; Ziff 2007; Vandenberghe, Nicoll, and Bredt 2005). Since then various structurally diverse auxiliary proteins have been associated with both AMPA and kainate glutamate-gated receptors (Tomita 2010; Greger, Watson, and Cull-Candy 2017; Galaz et al. 2015; Howe 2015).

In *C. elegans* the GLR-1 receptor, an AMPA glutamate receptor homologue, is required for glutamate-gated current response in a subset of interneurons that control avoidance behaviors. *C. elegans* expresses the evolutionarily conserved TARP proteins, STG-1 and STG-2 (stargazin-like protein), that act as auxiliary protein for GLR-1 receptors. These TARP homologues are required for regulation of only channel properties, but not trafficking. A second class of AMPAR auxiliary protein SOL-1 (***S***uppressor ***O***f ***L***urcher movement defect), has also been identified in *C. elegans* (Zheng et al. 2004). It is a type I transmembrane protein with an evolutionarily conserved CUB domains in the extracellular domain. SOL-1 mutants lead to loss of GLR-1 activity in *C. elegans* and it has been shown to regulate the rate of GLR-1 desensitization as well as its rate of recovery from desensitization. STG-1 and STG-2 with SOL-1, are essential for GLR-1 activity *in vivo* as well as in recombinant systems. SOL-1 can coassemble into a tripartite complex with STG-1 and GLR-1 in heterologous cells to reconstitute glutamate-gated currents, but interestingly, no mammalian homologue of SOL-1 has been identified so far (Walker et al. 2006; Wang et al. 2008). Another class of auxiliary protein, SOL-2, contributes to the GLR-1 complex and modifies the GLR-1 kinetics and pharmacology (Wang et al. 2012). SOL-2 is a transmembrane protein with two CUB-domains and is homologous to vertebrate NETO proteins, auxiliary proteins of kainate receptors. Overall, GLR-1 along with the associated TARP proteins, STG-1 and STG-2, interacts with a protein complex containing SOL-1 and SOL-2 at the plasma membrane. Loss of any of the component proteins results in significant alteration of channel properties. Additionally, the glutamate-gated currents are also affected by activity-dependent

changes in the relative numbers of auxiliary proteins present at the postsynaptic membrane (Wang et al. 2008; Wang et al. 2012; Walker et al. 2006; Zheng et al. 2004).

In contrast to glutaminergic receptors, only one auxiliary subunit, MOLO-1 (***mo***dulator ***o***f ***l***evamis***o***le receptor-1), has been identified for the nicotinic acetylcholine receptors in *C. elegans* (Boulin et al. 2012). It is an evolutionarily conserved single-pass transmembrane protein with a single extracellular globular domain, the TPM domain. The protein regulates the levamisole-sensitive nAChRs function through direct physical interaction. Null mutants of the protein results in impaired locomotion and loss of sensitivity to levamisole. MOLO-1 affects synaptic transmission in the worms and promotes channel opening in the heterologously expressed receptors. The existence of auxiliary subunits for nAChRs further amplifies their heterogeneity and pharmacological diversity. This also expands the repertoire of potential drug targets for nAChR complexes.

2.5 *Caenorhabditis elegans*

C. elegans is a small ~1mm long, transparent, free-living rhabditid nematode that was first described by Emile Maupus (Maupas 1900). The name has been derived from Greek *Caeno* (recent), *rhabditis* (rod-like) and Latin *elegans* (nice). The non-parasitic nematode is present in the soil, especially decaying organic material, where it survives by feeding on microbes such as bacteria. The wild-type *C. elegans* are sexually dimorphic; worms are either self-fertilizing hermaphrodites or males exhibiting differences in the reproductive functions and posterior anatomy (Chalfie 1984). The adult worms are bilaterally symmetrical with unsegmented cylindrical body shape that is tapered at the ends. The body plan consists of on outer tube (body wall) and inner tube separated by

pseudocoelomic space. The body wall consists of cuticle, hypodermis, excretory system, neurons, and muscles while the inner tube comprises the pharynx, gut and the reproductive system. The adult hermaphrodite has 959 somatic cells, 30% (302) of which are neurons. On the other hand, an adult male has 1031 somatic cells and 381 neurons; the extra neurons are dedicated mostly to reproductive behavior (White 1988; Sulston et al. 1983). Despite its anatomical simplicity, the worm displays a large repertoire of behaviors including locomotion, foraging, feeding, defecation, egg laying, sensory responses and complex behaviors including mating, social behavior and simple forms of learning. Remarkably, all these behaviors are accomplished by the small number of neuronal cell nuclei present in the adult nervous system (de Bono 2003; Bargmann 1993).

C. elegans is an extraordinarily powerful model system. The bacterivorous worm was used for the first time in early 1960's as a simple metazoan model to study developmental biology and neurobiology by Sydney Brenner (Brenner 1973). Over time this remarkable expression system has addressed many fundamental questions in multiple fields of biology concerning both parasites and humans (Leung et al. 2008; Teschendorf and Link 2009; Newman et al. 2017; Nussbaum-Krammer and Morimoto 2014; Alexander, Marfil, and Li 2014; Ma et al. 2018; Buckingham, Partridge, and Sattelle 2014; Katiki, Ferreira, et al. 2011; Geary and Thompson 2001). It has a number of features that foster its value as a biological research tool and enables experimental use. The small size allows large numbers of worms to be easily cultivated in a small space including microtiter plates. It can easily thrive on agar plates or in liquid media supplemented with a bacterial diet, making it convenient and economical to maintain in laboratory conditions. Furthermore, *C. elegans* is the only known multicellular organism

which can be stored indefinitely in liquid nitrogen making it possible to possess and archive an unlimited collection of mutants (Stiernagle 2006). The relatively short life cycle (~3.5 days), quick generation time with high progeny number (~300) and short life span (~ 2 weeks) renders it amenable to high-throughput manipulations. The transparent body of the non-parasitic nematode allows clear observation of all the body cells facilitating developmental and cell biology research. (Brenner 1973; John 1988; Sulston et al. 1983; Bürglin, Lobos, and Blaxter 1998; Riddle et al. 1997). Studies that utilize fluorescent proteins including *in vivo* electrophysiological recordings, mapping of synaptic and cell to cell contacts and optogenetic explorations are greatly aided by this physiological property (Kerr 2006; Husson, Gottschalk, and Leifer 2013; Goodman et al. 2012). The hermaphrodite nematode was the first multicellular organism to have its whole genome sequenced (Consortium 1998). The highly accurate, complete and well-annotated genome sequence and vast library of mutant strains makes *C. elegans* compliant to a plethora of well-established genetic manipulations and alterations (Kaletta and Hengartner 2006; Lamitina 2006). It is also possible to raise genetically identical populations without the risk of inbreeding depression from *C. elegans* as they reproduce by self-fertilization (Rieckher et al. 2009). Furthermore, the nervous system of *C. elegans* is small and tractable. It provides a unique opportunity to unfold the complex behaviors at the levels of neural circuitry and genes without the interference of pleiotropic effects that are often seen in other model organisms (Rankin 2002; Corsi 2006).

There has been an increased interest in identifying novel anthelmintic targets and developing new agents in the past few decades. But one of the major challenges

for helminth studies is posed by the complicated life cycles with reliance on host *in vivo* models, wide range of size and diverse activity of parasitic nematodes, lack of *in vitro* culture systems and high expenditure involved in maintenance of *in vivo* models for many parasitic species. The inherent simplicity and extensive toolkit available for *C. elegans* offers great promise for antiparasitic drug discovery. *C. elegans* not only shares morphological similarities with parasitic nematodes but displays sufficient conservation of biological characteristics and regulatory processes including protective cuticle, dauer stages, biochemical adaptations to extreme conditions, molting and reproduction that are divergent from mammalian host species. Parasitic nematodes also share physiological and pharmacological characteristics with *C. elegans* which is an important consideration for drug discovery (Holden-Dye and Walker 2014). The majority of the marketed anthelmintic drugs are effective against *C. elegans* which highlights the validity of the worm for parasitic nematode research (Burns et al. 2015; Kaminsky et al. 2008; Holden-Dye and Walker 2014). The value of this free-living nematode for comparative functional genomics and transgenesis studies has also been unequivocally demonstrated (Britton and Murray 2006). WormBase, founded in 2000, is an excellent comprehensive source for *C. elegans* biology, proteomics and gene information (http://www.wormbase.org). This centralized public online platform helps to prioritize hits for target identification. *C. elegans* has proven instrumental in providing insights into mechanism of action and resistance of various anthelmintic agents including cholinergic anthelmintics and avermectins (Kaminsky et al. 2008; Driscoll et al. 1989; Kaletta and Hengartner 2006; Lewis et al. 1980; Rufener et al. 2013; Dent et al. 2000; Jones, Buckingham, and Sattelle 2005; Fleming et al. 1997). The worm model has contributed decisively to our

understanding of parasite biology and its utility as a model organism for discovery of anthelmintics is undeniable.

2.5.1 *C. elegans* pharynx

C. elegans is a microbivores filter feeder and the feeding motion is under the control of the pharynx. It is a prominent feature in the head region that connects the buccal cavity and the intestine (Avery and Horvitz 1989; Albertson and Thomson 1976). The pharynx is an encapsulated myogenic neuromuscular organ with similarities to the vertebrate and invertebrate heart that pumps throughout the life of the worm. It undergoes a rhythmic cycle of contraction and relaxation that helps in drawing bacteria into the lumen, grinding and transporting them into the intestine. The feeding behavior is tightly regulated by a complex interplay of neurotransmitters, neuromodulators and electrically coupled muscles (Avery and Horvitz 1990; Albertson and Thomson 1976). Feeding is vital for survival and thus targeting this behavior in nematodes has pharmaceutical relevance. *C. elegans* is physiologically and pharmacologically related to many parasitic nematodes (Holden-Dye and Walker 2014). Thus, discovery of pharyngeal targets and associated drugs that inhibit feeding in this species should generalize to other nematode species of clinical importance.

2.5.2 Anatomy of *C. elegans* pharynx

Albertson and Thomson (1976) performed ultrastructural analysis, using serial section electron micrographs, to reconstruct the anatomy of the *C. elegans* pharyngeal cells and their respective neural circuitry. The pharynx is 100µm long tubular, bilobed structure with a diameter of approximately 20µm at its widest. It is connected to the buccal cavity at the anterior and to the intestine at the posterior (Figure 2.11A). The

entire structure is self-contained, encased in specialized basal lamina that isolates it from the pseudocoelom. It has a simple anatomy consisting of three functional units, namely, the corpus (divided into procorpus and metacorpus/anterior bulb), isthmus and the terminal/posterior bulb (Figure 2.11B). The pharyngeal lumen and buccal cavity are lined by cuticle and is continuous with the outer cuticle of the body. The lumen of the pharynx is triangular anteriorly and in the procorpus it becomes triradiate, with tubular apices. The procorpus is completely closed posteriorly while the metacorpus is slightly more open. The tubular apices reappear briefly at the anterior of the isthmus. The cuticle forms specialized structures that serves as sieves in the anterior of the procorpus and grinder in the terminal bulb. The pharynx has its own muscles, nervous system, gland cells and structural cells. The mature pharynx is composed 62 cells containing 80 nuclei which includes 20 muscle cells of anatomical types (37 nuclei) connected by 8 gap-junction, 9 marginal cells, 9 epithelial cells, 4 gland cells (5 nuclei) and 20 neurons of 14 anatomical types (Hall and Altun 2008).

The muscle cells are grouped into eight separate segments (pm1-8). These are arranged as eight consecutive rings encircling the pharynx. The corpus comprises of pm1 through pm4 forming the anterior part and its purpose is to draw in and trap the bacteria. The isthmus, the muscle pm5, is the middle part and regulates the flow of food from the corpus to the terminal bulb. The isthmus is surrounded by the large somatic nerve ring. The terminal bulb is formed from muscles pm6 through pm8 and connects to the intestine by the pharyngeal-intestinal valve or cardia. Pm6 and pm7 secrete a thick, ridged cuticle which forms the grinder which facilitates the breaking up of the bacteria (Albertson and Thomson 1976; Franks et al. 2006).

The pharyngeal muscle cells of each segment are separated from each other by three marginal cells. The nine marginal cells are arranged in sets of three cells (mc1-mc3). Together, the muscles cells and marginal cells form a single-cell-thick epithelial tube with trifold symmetry (two bilateral symmetrical left and right subventral sides, and one dorsal side). The muscle cells communicate by a network of desmosomes or gap junctions. These exist between muscles and marginal cells, and between neighboring marginal cells (Albertson and Thomson 1976). The gap-junctions are made up of innexins, pore forming proteins that express hexameric hemichannels. These hemichannels confer a high degree of electrical connectivity and are required for myogenic excitation and neuronal modulation (Altun et al. 2009; Phelan 2005; Franks et al. 2006). The gap-junctions divide the pharyngeal muscle membranes into apical surfaces which faces the lumen and the basal surfaces which faces the pseudocoelom. The longitudinal pharyngeal nerve cord is located in a deep groove formed by syncytial muscle cells on the basal side. The pharynx is not covered by an epithelial sheet but is surrounded by a narrow ring formed by nine epithelial cells that connects with the mouth opening (Albertson and Thomson 1976). These cells contain filaments that anchor the basement membrane to the cuticle. Two sets of gland cells, g1 and g2, distinguished by the fine structure of the cytoplasm are found in the second bulb of the pharynx. The g1 type have two cells with three nuclei and lamellar cytoplasm while the other set, g2, has two cells with two nuclei have clearer cytoplasm with vesicles. These cells open into the lumen via a short cuticular duct. The gland cells secrete vesicles just before hatching, at each larval molt and during feeding; the secretions may possibly

contain mucoids (Smit, Schnabel, and Gaudet 2008; Albertson and Thomson 1976; Hall and Hedgecock 1991).

The nervous system of pharynx is a self-contained, nearly autonomous system as it makes a connection with only one bilaterally symmetrical pair of extrapharyngeal neurons (Albertson and Thomson 1976). It is composed of 20 neurons, mostly unbranched unipolar and bipolar cells, and accounts for all chemical synapses onto pharyngeal muscle. Based on their physiological or behavioral functions they have been classified into five classes of motor neuron (M1-M5), six classes of interneurons (I1-I6), two neurosecretory motor neurons (NSM), a motor interneuron (MI) and a pair of neurons which innervate marginal cells (MC) (Figure 2.11C). These neurons are contained in folds of the pharyngeal muscle basal membrane, between the muscle and the basal lamina.

2.5.3 Feeding behavior in *C. elegans*

Feeding in C. *elegans* is achieved by a rhythmic pumping motion of the pharynx (Avery and Shtonda 2003; Doncaster 1962) (Figure 2.11D). The electrically coupled pharyngeal muscle cells pump throughout the life cycle of the worm except during molting or dauer larval stage. Normal feeding consists of two stereotyped motions: pumping, a contraction-relaxation cycle involving the corpus, anterior half of the isthmus and terminal bulb; and the posterior isthmus peristalsis. A pump represents a near-simultaneous contraction of the muscles of the corpus, anterior isthmus and terminal bulb followed by near-simultaneous relaxation. The corpus and anterior isthmus have two functions, one to trap food and the other to transport food. The pharyngeal lumen during the resting state is closed resembling a Y-shape. The contractile fibers of the

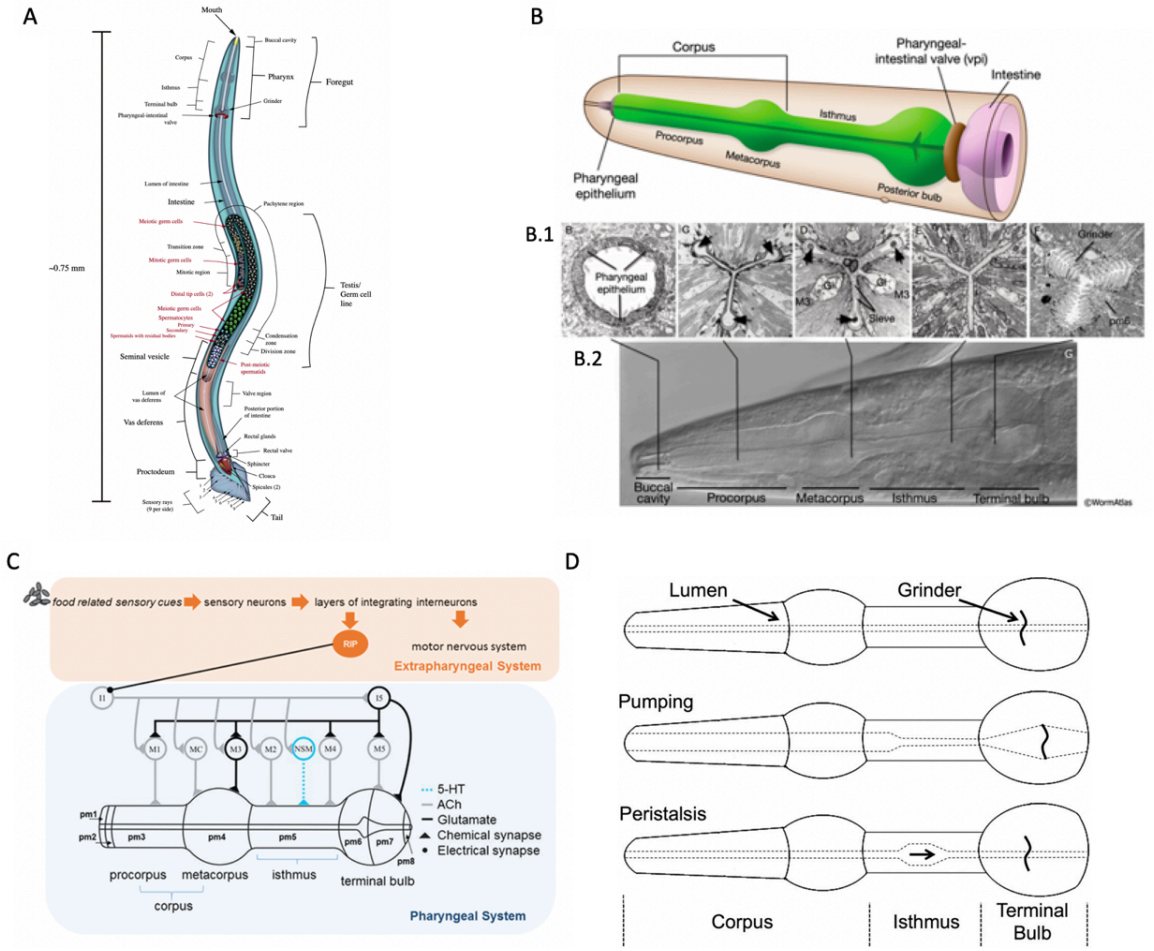

Figure 2.11 A. Anatomical drawing of a male *C. elegans* nematode (modified from https://en.wikipedia.org/wiki/File:C_elegans_male.svg). B. Graphic representation of the pharynx highlighting the corpus, the isthmus and the terminal bulb. Transmission electron microscope sections through various parts of the pharyngeal lumen are shown in B.1 along with the section levels indicated in the micrograph of the pharynx shown in B.2. The arrows in the procorpus section (*arrows*) indicates the channels seen at the three corners of the lumen. In the metacorpus section the two-ventral gland (Gl) openings and M3 cell bodies can be seen. Bacteria (*arrows*) are seen in channels while the rest of the lumen is structured as a sieve. The TEM section of the isthmus is shown at the level of the excretory duct. Grinder can be seen in the section of the terminal bulb grinder (modified from doi:10.3908/wormatlas.1.3). C. The image showing organization of the pharyngeal system. The contraction-relaxation cycle of the pharyngeal muscle is regulated by the pharyngeal interneurons and motor neurons. A subset of the 20 pharyngeal neurons is depicted along with their major neurotransmitter phenotype. The pharyngeal nervous system is connected to the extrapharyngeal system by a gap junction linking RIP (ring/pharyngeal interneuron) to I1. The neurotransmitters released from neurons within the pharyngeal system are also shown (Dallière et al. 2017). D. A diagram showing pumping behavior in *C.* elegans (anterior end is left). During a pump, food enters via the corpus, which is then transferred along the isthmus via posteriorly propagating peristaltic waves. The food is broken up by the cuticular grinder in the terminal bulb during the subsequent pump (Trojanowski, Raizen, and Fang-Yen 2016).

pharyngeal muscles are radially oriented; contraction pulls the lumen open to a triangular cross-section, sucking in liquid and suspended bacteria from outside. The posterior isthmus remains closed during this period of the cycle; it separates the corpus and anterior isthmus from the terminal bulb. The contraction of the terminal bulb muscles rotates the grinder plates which breaks up the bacteria and the food is returned to the lumen. Following contraction, there is a near-simultaneous relaxation of the muscles and the grinder returns to its resting position. This closes the lumen of the corpus and anterior isthmus, resulting in expulsion of liquid while retaining bacteria. The corpus contracts and relaxes as a unit but the anterior isthmus contracts in a wave that proceeds from anterior to posterior. In addition, these motions of the anterior isthmus are slightly delayed, resulting in a net posterior transport of the food particles during a pump (Seymour, Wright, and Doncaster 1983). During the next contraction and relaxation cycle, the trapped bacteria are carried in a posterior direction by the influx of liquid. With repeated pumps, the bacteria are transported further posteriorly in the isthmus. The second motion, isthmus peristalsis, is a posterior-moving peristaltic wave of contraction in the posterior isthmus that carries food accumulated in the anterior isthmus back to the terminal bulb and intestine. The isthmus peristalsis typically occurs every third to fourth pump following the completion of main relaxation (Avery and Horvitz 1987).

2.5.4 The pharyngeal muscle action potential

The pharyngeal pumping behavior is a consequence of a single muscle action potential. The pharyngeal muscles generate myogenic rhythm and the pharyngeal nervous system entrains the contraction rate and timing through neurotransmitter

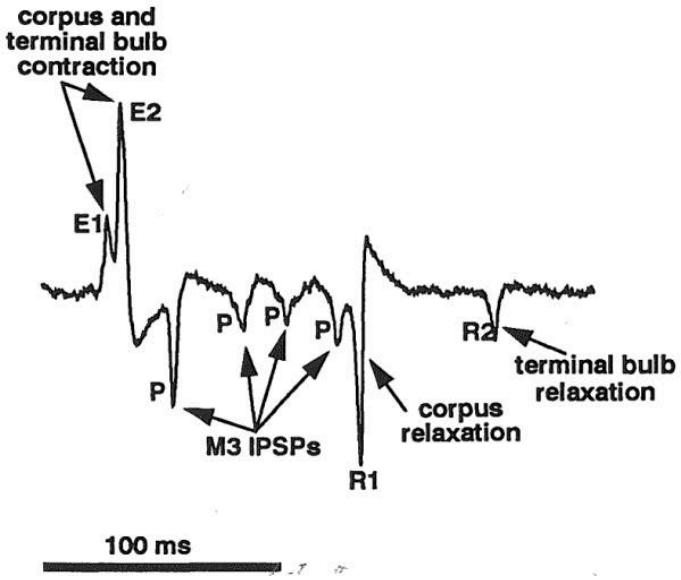

Figure 2.12 An image of a normal electropharyngeogram (EPG) from *C. elegans*. Different phases of the EPG are indicated (Avery, Raizen, and Lockery 1995).

release (Trojanowski, Raizen, and Fang-Yen 2016). The action potential generated by pharyngeal muscles are comparable to the vertebrate ventricular myocardium; they are long-lasting and have three phases, called E for excitation, P for plateau, and R for repolarization (Figure 2.12).

Raizen and Avery (1994) performed the first extracellular recordings, termed electropharyngeograms or EPGs; Avery, Raizen, and Lockery (1995) pioneered the technique to study pharyngeal excitation in *C. elegans*. EPGs provide information regarding the rate of pharyngeal pumping, duration of each pump and the activity of neurons within the pharyngeal nervous system. A typical EPG begins with excitation, viewed as a rapid rise in the membrane potential to 30-40 mV from the resting value of -40 to -50 mV. This E phase is divided into two components; the initial part (E1), which is a small excitatory postsynaptic potential associated with activation of MC motoneuron, immediately followed by a large depolarizing spike (E2). This is followed by a plateau phase which lasts for about 150 msec during normal wild-type pumping. A repolarizing

spike, R1, terminates the plateau phase with a drop in the membrane potential to more negative values than resting membrane potential. R1 represents the repolarization of the corpus, which is followed by a much smaller repolarizing spike, R2, associated with repolarization of the terminal bulb. Between E2 and R1, inhibitory postsynaptic potentials (I potentials) of varying amplitudes are often observed. Each contraction-relaxation cycle corresponds to a single muscle action potential. EPG amplitude and shape is dependent on the relative placement of the recording electrode and the resistance of the seal making it difficult to compare the magnitude of pharyngeal potentials between individual worms (Cook, Franks, and Holden-Dye 2006).

2.5.5 Role of pharyngeal nervous system in regulation of feeding

The pharyngeal nervous system is not essential for pumping, but as with the heart, serves a modulatory role. Using laser ablation, extensive analysis of mutants and electrophysiological recordings, key neurons required for efficient feeding have been identified (Figure 2.10C). These include M3, M4, MC, I1, I5 and NSM; physiological or behavioral functions have been defined for these neurons (Avery and Horvitz 1989, 1987; Avery 1993b; Raizen, Lee, and Avery 1995).

M3 motoneurons are a bilaterally symmetric pair of motor neurons, which primarily synapse onto the pm4 cells. They have free subcuticular endings in the metacorpus, suggesting they may be proprioceptive sensory neurons (Albertson and Thomson 1976). M3s receive chemical synaptic inputs from I2, I3, I4, I5, M1 and NSM but do not appear to have strong synaptic inputs onto any neurons. These motoneurons are glutamatergic and generate fast inhibitory post-synaptic potentials (IPSPs) in response to corpus muscle contraction and are responsible for the small negative spikes in EPG

(Avery 1993b; Raizen and Avery 1994). They regulate the pumping duration by triggering the end of a pump by initiating pharyngeal relaxation which is important for effective trapping of bacteria (Avery 1993b). These actions are mediated through glutamate-gated chloride channels formed by *avr-15* (*av*ermectin *r*esistant gene). Mutations in *avr-15* eliminates both the M3 transmission as well as the responses to glutamate. A chloride-dependent depolarizing current dependent on *avr-15* was observed in response to exogenously applied glutamate during intracellular recordings from pharyngeal muscles (Dent et al. 2000; Dent, Davis, and Avery 1997). Heterologously expressed avermectin-sensitive glutamate channels from *C. elegans* were found to be irreversibly opened by avermectin (Cully et al. 1994b).

M4 motorneuron cell bodies lie at the level of nerve ring and they form synapses with the posterior part of the isthmus, pm5 and g1 gland cells. M4 receives chemical inputs from I3, I5 and I6. This motorneuron is necessary for posterior isthmus peristalsis and regulate the swallowing motion of the isthmus (Albertson and Thomson 1976). M4 ablated worms fail to grow since they swallow little to no food as the posterior isthmus remains relaxed and the lumen closed. However, the worms still continue to pump, consequently the anterior isthmus and corpus become stuffed with the concentrated bacteria (Avery and Horvitz 1989). M4 are cholinergic as they stain with antibodies against UNC-17 (Chiang et al. 2006; Alfonso et al. 1993). However cholinergic antagonists and mutations affecting nicotinic ACh receptors only partially block M4 function. These motorneurons also contain large dense-core vesicles at neuromuscular junctions and also express the 5-HT receptor, SER-7b and the glutamate receptor subunit, GLR-8 (Brockie et al. 2001; Hobson et al. 2006; Albertson and

Thomson 1976); this suggests acetylcholine is probably not the only neurotransmitter in M4s.

NSMs are a pair of bipolar neurons with cell bodies at the level of the metacorpus. The axons of these motoneurons run in a posterior direction through the isthmus but terminate before reaching the terminal bulb, making contact with pm5. NSMs make a chemical synaptic contact with I6 and M3 and receive a chemical input from I1, I2, I3, I4 and I6. NSMs have putative sensory endings at the boundary between the corpus and isthmus. The NSM axons are swollen with varicosities and contain mixed sized vesicles and the processes of these neurons run in close apposition to the pseudocoelom over most of their length (Albertson and Thomson 1976). NSMs contain the neurotransmitter, serotonin (Horvitz et al. 1982). It is possible that these neurons play neurosecretory and motor function, communicating the presence of food in the pharyngeal lumen (Albertson and Thomson 1976). Exogenous application of the neurotransmitter depresses locomotion, stimulates egg laying and pumping in hermaphrodite worms (Horvitz et al. 1982; Croll 1975b). All of these responses are observed when the bacteria are present in the environment (Croll and Smith 1978; Croll 1975a), making NSMs a possible candidate for mediating the effects of endogenous serotonin. However, laser killing of NSMs produces little measurable effects on pharyngeal pumping suggesting these neurons are redundant for this function (Avery, Bargmann, and Horvitz 1993).

MC are excitatory neurons with cell bodies located at the level of the metacorpus. Their axons are short, the posterior ones terminating at the very anterior end of the isthmus. MCs make electrical connections with M2 and receive chemical synaptic inputs

from M1 and I1. MCs do not synapse on muscle but onto the marginal cells, mc2, suggesting that the signal spreads through gap-junctions (Albertson and Thomson 1976). Like M3 motorneurons, MC may be a sensorimotor neuron as their putative free subcuticular endings, between the procorpus and metacorpus, might be able to sense bacteria (White et al. 1986). MC behaves as a motorneuron, producing a fast depolarization of muscle membrane potential (excitatory postsynaptic potential, EPSP), which triggers a muscle action potential. Thus, it acts as a neurogenic pacemaker for rapid pharyngeal pumping and is the sole neuron responsible for this type of behavior (Raizen, Lee, and Avery 1995). MCs are required for maintaining optimal rate and frequency of pharyngeal pumping rate. Following MC ablation, the pumping becomes irregular, slowing down from around 250 to 50 pumps per minute and resulting in starved adults (Avery and Horvitz 1989). MC neurons are cholinergic; mutations disrupting acetylcholine synthesis (*cha-1*) or packaging (*unc-17*) produce a pumping defect similar to MC ablation. Acetylcholine and cholinergic agonists produce excitation of the pharyngeal muscles and nicotinic blocker, curare, competitively blocked the MC neuromuscular transmission. In addition, genes encoding for nAChR subunit, *eat-2*, and a protein required for nicotine response in pharyngeal muscles, *eat-18*, mimic the phenotype of worms with MC loss (Raizen, Lee, and Avery 1995).

Interneurons (I1 and I5) have been shown to regulate the functioning of MC, M3 and M4, hence influence feeding. The cell bodies of I1s are located immediately anterior to the metacorpus and their axons project onto pm1 and pm2. I1 neurons receive the gap junctions from the somatic system RIPs (ring/pharynx interneuron), thus connecting the pharyngeal and the extrapharyngeal nervous system (Avery and Thomas

1997). These interneurons make chemical synapses with MC and affect the rate of pumping in the absence of bacteria (Franks et al. 2006; Albertson and Thomson 1976; Trojanowski et al. 2014). Trojanowski et al. (2014) demonstrated that I1s endogenously excite pumping via both the MCs and the M2s in a state-dependent manner. I5s are sensory interneurons and have a large cell body on the ventral side of the terminal bulb. It forms synapses on M3 (Albertson and Thomson 1976). In I5 ablated worms, the pump duration was decreased due to faster relaxation. Killing both I5 and M3 resulted in an increase in pump duration due to delay in relaxation, indistinguishable from that caused by M3 ablation alone. Thus, I5 increased the pump duration by inhibiting M3 motoneuron (Avery 1993b).

2.5.6 Role of neurotransmitters and biogenic amines in feeding

The pharyngeal nervous system contains classical neurotransmitters (acetylcholine, glutamate) and biogenic amines (serotonins, dopamine, octopamine, tyramine) which assist with various aspects of feeding behavior.

Acetylcholine is contained in at least eight pharyngeal neurons, M1, M2s, M4, M5, MCs and I6 (Franks et al. 2006). As discussed above, it is mainly MCs that play key roles in the cholinergic stimulation and regulation of pharyngeal functions (Avery and Horvitz 1989, 1987). Of the 32 nicotinic acetylcholine receptor subunits identified in *C. elegans* so far, EAT-2 has been identified in the pharynx (Raizen, Lee, and Avery 1995). It is a non-alpha subunit localized near the junction of pm4 and pm5, also the site where MC synapses (McKay et al. 2004). Acetylcholine released from stimulated MCs increases the pharyngeal pumping rate by activating EAT-2 containing nAChRs on pm4. *eat-2* mutants have a reduced pumping rate in the presence of food, similar to MC

ablated worms (McKay et al. 2004; Raizen, Lee, and Avery 1995). Another protein encoded by *eat-18* is expressed in pharyngeal muscle and M5. Eat-18 is a small single-pass protein with no homology to previously characterized proteins. The null mutants of this protein also generate a phenotype indistinguishable from the worms which had MC motorneurons killed. Another α-7-like nAChR subunit encoded by *acr-7* is present in the pharyngeal muscles and is sensitive to anti-psychotic drugs (Saur et al. 2013).

Five pharyngeal neurons, viz, M3, NSM and I5 contain **glutamate** (Lee et al. 1999). Invertebrates express unique glutamate-gated chloride channels (GluCls) that exhibit fast inhibitory glutamatergic transmission resulting in hyperpolarization. GluCls are targets of extremely potent class of anthelmintics agents, macrocyclic lactones which includes avermectins (Arena et al. 1992; Cully et al. 1994b). These channels are most closely related to mammalian glycine receptors and distantly related to the GABA-gated chloride channels (Vassilatis et al. 1997). Out of the 6 genes that encode GluCl subunits in *C. elegans* four, *glc-1*(encoding GLC-1), *glc-2* (encoding GLC-2), *avr-14* (GluClα3A and GluClα3B) an *avr-15* (encoding AVR-15/GluClα2) are present in pharynx. *avr-15* is localized in the pm4 (metacorpus) and pm5 (isthmus) pharyngeal muscle cells. As already mentioned before, the actions of M3 released glutamate are mediated through ivermectin-sensitive GluClα2/AVR-15 (Dent, Davis, and Avery 1997). Mutations in *avr-15* renders the pharynx insensitive to ivermectin (Pemberton et al. 2001). Heterologously expressed GLC-2, assembled into a homomeric glutamate sensitive and ivermectin insensitive channels (Cully et al. 1994b). The expression of this GluCl subunit is limited to the pm4 muscle cells in the worms (Laughton, Lunt, and Wolstenholme 1997). GLC-1 also expresses as a homomeric channel (Cully et al. 1994b). In contrast to other GluCls,

glutamate binds to GLC-1 only after ivermectin has already bound. *avr-14* is expressed in M1 and M4 but is not present in the pharyngeal muscles. This gene encodes for splice variants, GluClα3A and GluClα3B. However, *avr-14* null mutants are still responsive to ivermectin and indistinguishable from wild type worms (Pemberton et al. 2001). In terms of function, glutamate regulates the pharyngeal pumping, sensory perception and locomotion in the worms (Dent, Davis, and Avery 1997; Dent et al. 2000). AVR-15 functions to inhibit pharyngeal muscle contractions during pharyngeal pumping (Dent et al. 2000; Dent, Davis, and Avery 1997; Cook et al. 2006). Additionally, mutations in or knockdown of *avr-15* or *glc-1* increases the frequency with which worms change direction. AVR-15 along with AVR-14 mediates mechanosensory inhibition of pharyngeal pumping (Keane and Avery 2003).

Biogenic amines, serotonin (5-hydroxytryptamine, 5HT), dopamine, octopamine and tyramine, function as neurotransmitters or neuromodulators (Sanyal et al. 2004; Horvitz et al. 1982; Alkema et al. 2005; Sulston, Dew, and Brenner 1975). They modulate a variety of worm behaviors in response to the surrounding environment. **5-HT** is found in the NSM and I5 neurons (Albertson and Thomson 1976; Horvitz et al. 1982). Exogenous serotonin stimulates pharyngeal pumping from around 40 pumps per minute to over 250 pumps per minute (Avery and Horvitz 1990). Endogenous reduction of 5-HT decreases pharyngeal pumping rate (Sze et al. 2000). Mutants of *tph-1*, a gene coding for tryptophan hydroxylase, are viable but deficient in 5-HT synthesis which alters the feeding behavior. The worms have low average pumping rate in the presence of food (Sze et al. 2000). Hobson et al. (2006) reported that *tph-1* mutants can pump at a rate comparable to wild type animals, but the pumping behavior is irregular with inability

to sustain a fast pump rate. Therefore, 5-HT is required to maintain a sustained high pumping rate in the presence of food and not for basal pumping. 5-HT mediates its actions through three GPCR coupled (SER-1, SER-4 and SER-7B) and one serotonin-gated chloride channel (MOD-1) receptors, expressed in pharyngeal neurons and muscles (Hamdan et al. 1999; Ranganathan, Cannon, and Horvitz 2000; Hobson et al. 2003). Serotonergic transmission through SER-7 is essential for stimulation of pumping as *ser-7* mutants fail to exhibit 5-HT induced feeding. The presence of bacteria can still stimulate pumping (Hobson et al. 2006). This shows serotonin is not the only signal required to induce feeding and the stimulation of feeding behavior has a complex neuronal basis (Franks et al. 2006). **Dopamine** is another biogenic amine that affects feeding behavior in *C. elegans*. Dopamine signaling assists the worms with efficient food search (Sawin, Ranganathan, and Horvitz 2000). Exogenous dopamine application does not affect the pumping rate on food (Barros et al. 2014). Both **octopamine and tyramine** are not synthesized in the pharyngeal nervous system, but their exogenous application reduces pumping rate (Horvitz et al. 1982; Alkema et al. 2005; Rex et al. 2004; Rogers et al. 2001), therefore they have a neurohormonal function. Tyrosine is the precursor for synthesis of tyramine and tyramine-β-hydroxylase (TBH-1) converts tyramine to octopamine. Octopamine mediates its effects through suppression of M3 activity which increases the action potential duration. SER-3 is the octopamine receptor found on the pharyngeal muscles and *ser-3* mutants have a slightly lower pumping rate than wild type worms in the absence of food (Carre-Pierrat et al. 2006). Tyramine inhibits pumping through SER-2 receptors present on NSM neurons, pm1 and pm6 muscles (Rex et al. 2004); TYRA-2 receptors expressed in MC and NSM neurons (Rex et al. 2004).

2.6 *Ascaris* spp. pharynx

Similar to *C. elegans*, the pharynx of *Ascaris* is essential for feeding and regarded as an exploitable target for development of therapeutics. An example is the macrocyclic lactones which target pharyngeal ion channels and interrupt pharyngeal processes (Brownlee, Holden-Dye, and Walker 1997; Wolstenholme and Rogers 2005). *Ascaris* spp. are parasitic nematodes with a limited glycogen reserve. They feed on the host tissue and fluids to meet their energy demands. A functional failure of the pharynx affects the survival of the worms as starving and sluggish worms are removed from the host during intestinal movements.

2.6.1 Anatomy of *Ascaris* spp. *pharynx*

The pharynx of *Ascaris* is thick walled, 8-11 mm long, and up to 1 mm wide hollow cylindrical structure, developed from the fusion of about 30 cells (Brownlee et al. 1995; Goldschmidt 1904). It is a musculo-glandular motile segment of the digestive system, externally surrounded by the cuticle along with hypodermis and the somatic muscle layer (Figure 2.13A). The lumen of the pharynx is tripartite and derived from the cuticle (del Castillo and Morales 1967a). The muscular portion of the pharynx consist of two types of fibrillar components, the contractile ordinary muscle fibers and the marginal muscle fibers, arranged like the segments of an orange. The marginal muscle fibers are heavily concentrated at the apices of the triradiate lumen (Goldschmidt 1904; Hsü 1929; Mapes 1965). The muscle fibers are transversely oriented to the long axis of the esophagus arranged as bundles of thick and thin filaments (Reger 1966). This arrangement suggests that the esophagus function is mechanically analogous to the vertebrate diaphragm (del Castillo and Morales 1967a). The non-myofibrillar cytoplasmic

regions of the myoepithelium contain mitochondria, endoplasmic reticulum, and bundles of tightly packed, filaments. The glandular cells are interspersed between the myoepithelial cells, producing digestive enzymes. There are two one-way valves present at both ends, anterior and posterior, ensuring unidirectional flow of the material. The function of the tube-shaped pharynx is to pump food against the inner peudocoelomic pressure and prevent gut contents from being regurgitated (Bennet-Clark 1976).

2.6.2 Pharyngeal peristalsis in *Ascaris*

Unlike *C. elegans,* the pharynx of *Ascaris* is opaque and a simple cylindroid with no distinct bulbs. The contraction of the pharyngeal musculature in the parasitic nematode undergoes rhythmic peristalsis, rather than two stage pumping seen in *C. elegans* (Mapes 1966; Harris and Crofton 1957b) (Figure 2.13B). The pharyngeal peristalsis starts at the anterior tip, considered as the pacemaker, and travels all the way to end of the pharynx. The initiation of the rhythmic peristaltic motion is regulated by either myogenic or enteric nervous system activity (Brownlee et al. 1995). The depolarization of the muscles of the pharynx results in progressive contraction waves which move at an average speed of 4 cm/sec. The contraction of the radially oriented muscle fibers, results in the expansion of the outer diameter of the cell. This converts the narrow lumen into a wide triangular canal with a subsequent decrease in pharyngeal pressure, thus facilitating suction of food through a one-way valve near the mouth. Rapid relaxation of the contractile fibers due to repolarization closes the lumen and results in increased internal pressure sufficient to overcome hydrostatic pseudocoelomic pressure. This forces the contents of the lumen to move into the intestine through a second one-way valve. The process of contraction and relaxation is known as a “pump”. A number of successive

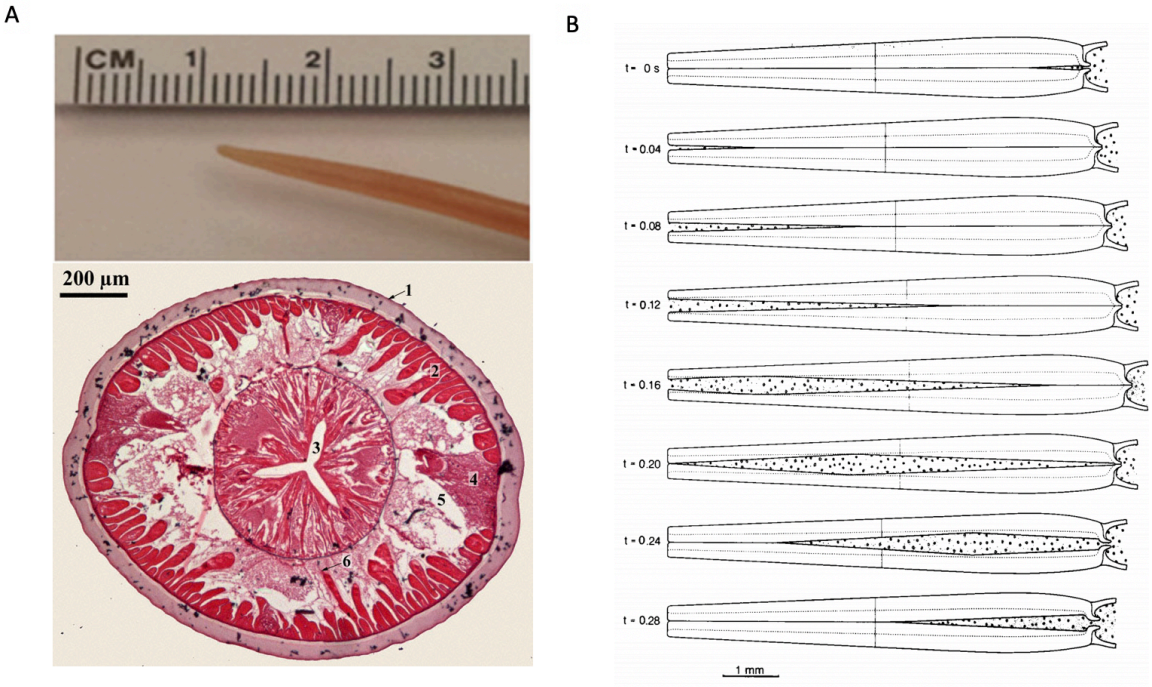

Figure 2.13 A. Photograph (top) of the head region of female *A. suum* (with cm scale above the worm). Image (bottom) showing cross-section of *A. suum* pharynx. The cuticle (1), muscle cells (2), pharynx with triradiate lumen (3), lateral chord (4), pseudocoel (5) and nerve chord (6) are indicated (modified from https://www.vcbio.science.ru.nl/en/image-gallery/show/labels/print/AN0013/). B. Diagram showing various stages and different time points of repeating cycle of pharyngeal peristalsis (approximately every 0.32 s) of *Ascaris* pharynx (Sauders and Burr 1978). At any point pharynx never opens simultaneously at both anterior and posterior ends.

pumps are needed for feed intake and transport in nematodes which is defined as pharyngeal pumping behavior. At any stage of pharyngeal peristalsis, the lumen of the pharynx never opens fully at both ends (Sauders and Burr 1978).The rate of peristaltic cycles in an intact *Ascaris* pharynx is 4 pumps/sec, while an isolated pharynx shows 2.5 pumps/sec (Sauders and Burr 1978; Mapes 1966).

2.6.3 Electrophysiology of the *Ascaris* pharyngeal muscle

The *Ascaris* pharynx behaves as a single tube-shaped cell functionally with no appreciable barriers to impede the flow of current within the cytoplasm. Electrophysiology of the pharyngeal muscles has been performed by del Castillo and

Morales (1967a, 1967b); Byerly and Masuda (1979) using current-clamp and voltage-clamp techniques. It has a resting membrane potential of -35 mV which is dependent on the extracellular anions and pH fluctuations. Increasing the external pH (7 to 8) increased the resting potential by about 4 mV, while lowering the pH (7 to 6) resulted in depolarization of about 20 mV. Unlike somatic muscle cells, the pharyngeal action potential exhibited two different regenerative, all-or-nothing mechanisms and is occasionally spontaneous (del Castillo and Morales 1967a). One of the processes result in the positive overshoot (depolarization) caused by the influx of cations which are probably sodium and calcium. The potential reaches a plateau and remains depolarized from 150 msec to several seconds. The depolarization can reach as high as 18 mV and results in the long-lasting shortening of the myofibrils associated with the opening of the lumen. The second regenerative process results in a unique negative-going action potential (as low as -108 mV) caused by a potassium spike. These K^+ currents are strikingly analogous to the classical Na^+ currents of the vertebrate skeletal muscle and nerve, except for inverted voltage dependencies. The intense negative spike drives the return from the plateau to the resting potential and is a relatively fast process. In the pharyngeal muscles, the negative-spike is associated with the rapid relaxation of the muscles and closing of the lumen. Both the prolonged positive-going action potential which maintains the contractions and the rapid termination by the negative-going action potential improve the efficiency of the pharynx as a pumping organ. Byerly and Masuda (1979) described a depolarizing postsynaptic potential (-10 mV reversal potential) which triggered the positive-going spike and a hyperpolarizing postsynaptic potential (-40 mV reversal potential) that activates the negative-going action potential when the membrane is depolarized. The

transition from one regenerative potential to another may be dependent on a transmitter and the associated receptor. The pharmacology of the receptor involved has not been explored yet but might be a potential target site for future anthelmintics.

del Castillo and Morales (1967a, 1967b) demonstrated the existence of membrane spaces within the cytoplasm of pharyngeal muscle. These spaces were proposed to behave as the tubular systems of the muscle cells in vertebrates that spread the action potential into the cytoplasm. Electrophysiological investigation using a microelectrode revealed that the recorded signals in these spaces resembled extracellular action potentials. The large intracellular spikes observed in the pharyngeal muscle cells are instead replaced by small transients. Thus, these cytoplasmic spaces are in communication with the external solution and separated from the cytoplasm by an active membrane which can generate action potentials. It is possible that these spaces are either invaginations of the surface membrane or interstices between the cells that originally form the pharynx.

2.6.4 Nervous system of *Ascaris* pharynx

The pharyngeal-sympathetic nervous system provides the innervation to the *Ascaris* pharynx. It is formed by the nerve ring located at the posterior end of the pharynx. The three longitudinal nerve strands, dorsal and a paired sub-ventral cords, extend anteriorly from the nerve ring to innervate the pharyngeal muscle (Sauders and Burr 1978; Goldschmidt 1910). The dorsal nerve is shorter and contain two nerve cells while the sub-ventral cords have four nerve cells each (Goldschmidt 1910). The longitudinal nerve cords are joined together by 2-3 cross-connecting commissures. These nerve cords spread across the pharyngeal musculature to provide widespread innervation,

and in turn, coordinate the muscular and glandular activity of the pharynx. The pharyngeal nervous system is chemically complex. It is regulated by cholinergic (excitatory), glutamatergic (inhibitory) and serotoninergic (excitatory) systems. In addition, sensory neurons of the pharyngeal nervous system have GABAergic components (inhibitory) and peptidergic secretory components (Brownlee, Fairweather, and Johnston 1993; Brownlee et al. 1996; Brownlee et al. 1995).

2.7 Plant-based compounds therapeutic compounds

Plants have been a remarkable source of medicinal products for the treatment of a wide spectrum of diseases. Various ancient records reveal the importance of the botanical compounds in sophisticated traditional pharmacopeia. The earliest records which date back to 2600 BCE in Mesopotamia, describes the use of approximately 1000 plant-derived substances (Borchardt 2002). "Ebers Papyrus" (1500 BCE), the Chinese Materia Medica (1100 BCE) (Huang 1998) and the Indian Ayurvedic system (before 1000 BCE, Charaka; Sushruta and Samhitas) (Kapoor 1990) also document the medical relevance of herbal products. Plant-based systems continue to be indispensable in healthcare even today. Natural products are important source of pharmaceutical agents as well as leads to bioactive molecules. Many therapeutics, including codeine, quinine, artemisinin, digitoxin, morphine, paclitaxel, galantamine, used for various medical conditions in modern pharmacotherapy are plant-based (Newman, Cragg, and Snader 2000; Newman and Cragg 2016; Samuelsson 2004; Butler 2004). An estimated four billion people living in the developing world rely on phytomedicine for primary pharmaceutical care (WHO 2005). Even in the developed part of the world, herbal remedies in the form of complementary and alternative medicine (CAM) are being widely adopted (Anquez-

Traxler 2011; Calapai 2008). In addition to exhibiting a wide array of medicinal properties, plant-based compounds are eco-friendly and affordable. Most of the plants and their products are also readily available in the tropical and sub-tropical regions. In case of issues with the supply of source material, recent advances and novel scientific resources can be used for organic synthesis of phytocompounds.

Phyotherapeutics have been used by traditional healers to treat parasitic infections and improve performance of livestock for eons. Different parts of plants such as garlic, onion, mint, walnuts, dill, parsley, male fern *Dryopteris filixmas* and *Artemisia* spp. and thyme have been used to treat helminth parasitism (Ferrell 1914; Lamson and Ward 1932; Guarrera 1999). The evidence of antiparasitic activity of phytocompounds is based on anecdotal observations and the bioactive compounds have not been fully identified. Therefore, controlled scientific experimental studies are ongoing to aim to verify and quantify the clinical plant activity, with many reports of research-based validation of the anthelmintic activity of several plants (Jaradat et al. 2016; Bissinger et al. 2014; Kaplan et al. 2014; Katiki, Chagas, et al. 2011; Trailović et al. 2015). The identification and validation of botanical compounds may accelerate the anthelmintic drug discovery and development and also mitigate the pressure on the limited classes of anthelmintic drugs available.

2.7.1 Monoterpenoids as potential anthelmintic agents

Essential oils are the aromatic and volatile organic products of secondary metabolism in plants. The term “essential oil” was coined in the 16th century by Paracelsus von Hohenheim, a swiss physician and alchemist (Guenther 1948). It is derived from Latin phrase “*Quinta essentia*” meaning “the effective component of a

drug'. The composition of essential oils is extremely complex and diverse as they contain compounds of several different functional-group classes. The insecticidal and nematocidal effects of essential oils and their active principles is well documented (Coskun et al. 2008; Camurca-Vasconcelos et al. 2007; Macedo et al. 2009; Cetin et al. 2009; Enan 2005; Panella et al. 2005; Kim et al. 2008). Thymol, the principal component of thyme (*Thymus vulgaris L.*) and santonin which is derived from buds of the *Artemisia* spp. were used in the late nineteenth and early twentieth century for the treatment of ascarids and hookworms infestations (Ferrell 1914; Kaplan et al. 2014; Lamson and Ward 1932). Ascaridol, a peroxide monoterpene, found in wormseed oil (*Chenopodium ambrosioides*), was described as 'one of the best-known anthelmintic". It was used for the treatment of several roundworm and hookworm infections in man and animals (Lamson and Ward 1932).

Terpene biomolecules, particularly monoterpenes and sesquiterpenes, are the major constituents of essential oils. Many of the monoterpenoid compounds have been identified as promising anthelmintic compounds (Andrés et al. 2012; Ntalli et al. 2010; Avato et al. 2017). Jaradat et al. (2016) demonstrated the anthelmintic effects of *Thymus bovei* essential oil, which is mainly composed of monoterpenoids (~90%), against gastrointestinal roundworms. Four monoterpenoid compounds (α-pinene, linalool, p-cymene, and thymol) exhibited antinematodal activity when used alone and in combination (Bissinger et al. 2014). Lei et al. (2010) also reported *in vitro* nematocidal activity of thymol and carvacrol (major components of thyme and oregano essential oils) against *Caenorhabditis elegans* and *Ascaris suum*. Carvacrol, a phenolic monoterpene found in *Origanum* spp. and *Thymus* spp., and carvacrol acetate, less toxic synthetic

derivative of carvacrol, exhibited *in vitro* and *in vivo* anthelmintic activity against *Haemonchus contortus* (Andre et al. 2016). Trailović et al. (2015) also demonstrated the potential anthelmintic effects of carvacrol in *A. suum*. Monoterpenoids produce the anthelmintic effects by targeting the nervous system of nematodes through the invertebrate-specific G protein-coupled receptors for octopamine and tyramine, acetylcholine esterase, nicotinic acetylcholine receptors and ionotropic gamma amino butyric acid (GABA) receptors (Enan 2005; Lei, Leser, and Enan 2010; Trailović et al. 2015; Enan 2001; Tong et al. 2013; Tong and Coats 2012; Park et al. 2003; Miyazawa, Watanabe, and Kameoka 1997; Höld et al. 2000). Additionally, these compounds can bind to multiple target sites, which can slow down the development of drug resistance (Theis and Lerdau 2003).

CHAPTER 3. EAT-18 IS AN ESSENTIAL AUXILIARY PROTEIN INTERACTING WITH THE NON-ALPHA NACHR SUBUNIT EAT-2 TO FORM A FUNCTIONAL RECEPTOR

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3.1 Abstract

Nematode parasites infect approximately 1.5 billion people globally and are a significant public health concern. There is an accepted need for new, more effective anthelmintic drugs. Nicotinic acetylcholine receptors on parasite nerve and somatic muscle are targets of the cholinomimetic anthelmintics, while glutamate gated chloride channels in the pharynx of the nematode are affected by the avermectins. Here we describe a novel nicotinic acetylcholine receptor on the nematode pharynx that is a potential new drug target. This homomeric receptor is comprised of five non-α EAT-2 subunits and is not sensitive to existing cholinomimetic anthelmintics. Importantly, this is the first report of successful heterologous expression of a non-α nicotinic subunit into a functional homomeric ligand-gated cation selective ion-channel. We found that EAT-18, a small transmembrane protein, is a novel auxiliary subunit protein essential for functional expression of the receptor. It directly interacts with the mature receptor and different homologues alter the pharmacological properties. Thus we have described not

only a novel potential drug target but also a new type of obligate auxiliary protein for nAChRs.

3.2 Introduction

Nematodes are multicellular organisms that exhibit diverse and complex physiological behaviors. These functions are controlled by a neuromuscular system that employs a large repertoire of highly regulated transporters, neurotransmitters, peptides and ion channels, which all contribute to homeostatic cell-cell communication (White et al. 1986; Wolstenholme 2011). Nicotinic acetylcholine receptors (nAChRs) are pore-forming membrane proteins belonging to the cys-loop ligand-gated ion channel superfamily. They are conserved throughout metazoan evolution and characterized by a pentameric subunit organization. nAChRs facilitate rapid ionotropic neurotransmission thereby controlling various physiological behaviors including reproduction, navigation, feeding, and locomotion (Greenberg 2014).

Nematode nAChRs, especially those found on somatic muscle, are targeted by the cholinergic anthelmintic drugs (Robertson, Buxton, and Martin 2013; Robertson, Bjorn, and Martin 2000; Buxton et al. 2014; Xiao et al. 2005; Greenberg 2014). These drugs help alleviate the nematode parasite burden of the 1.5 billion people affected globally as well as mitigate the threat to global food security caused by nematode parasites of livestock (WHO 2018; Abongwa, Martin, and Robertson 2017). Nematodes have a greater number (≥29) of nAChR subunits than vertebrates (17); variation in stoichiometry and subunit composition leads to diverse pharmacological sensitivities which makes them attractive anthelmintic targets (Holden-Dye et al. 2013; Buxton et al. 2014; Jones and

Sattelle 2004; Millar 2008). The introduction of recently discovered drugs such as amino-acetonitrile compounds (monepental) and spiroindoles (derquantel) which target nematode nAChRs further highlight their importance in drug discovery (Kaminsky et al. 2008; Robertson et al. 2002; Little et al. 2011).

All nAChR ion-channels are composed of five subunits forming a central ion conducting pore and can be either homomeric (one α subunit) or heteromeric (multiple subunits with at least 2 α subunits). Various chaperone/ancillary proteins, including RIC-3 (resistance to inhibitors of cholinesterase), UNC-50 (uncoordinated-50) and UNC-74 (uncoordinated-74), are required for correct folding, assembly of individual subunits into pentamers and trafficking of the mature nAChRs in a subtype dependent manner (Millar 2008; Haugstetter, Blicher, and Ellgaard 2005; Boulin et al. 2008). In addition, several structurally unrelated auxiliary subunit proteins have been identified for various ionotropic receptors (Schwenk et al. 2010; Tomita and Castillo 2012; Yan and Tomita 2012; Ben-Yaacov et al. 2017). These proteins are non-pore forming and interact directly with the receptor subunits to modulate channel properties. They do not exhibit any channel activity on their own and are required for certain aspects of *in vivo* channel function (Yan and Tomita 2012). Boulin et al. (2012) identified the first auxiliary subunit for nAChRs, MOLO-1 (modulator of levamisolereceptor-1), that regulates biological and biophysical properties of the L-type nAChRs in *C. elegans*. This demonstrates that nAChRs are tractable to regulation by auxiliary proteins contributing to biological and pharmacological diversity of nAChR subtypes.

In nematodes, the pharynx is a neuromuscular organ that undergoes rhythmic peristalsis to ingest food and is thus crucial for survival (Seymour, Wright, and Doncaster

1983; Albertson and Thomson 1976; Brownlee et al. 1995). Pharyngeal peristalsis is under the control of rhythmic activation by excitatory (cholinergic) and inhibitory (glutamatergic) motor neurons innervating the pharyngeal muscle (Martin 1996; Brownlee, Holden-Dye, and Walker 1997). Glutamate-gated chloride channels in the nematode pharynx have been exploited as targets for the avermectins (Tompkins, Stitt, and Ardelli 2010; Wolstenholme and Rogers 2005; Cully et al. 1994a), however little is known about the cholinergic receptors in this tissue. Through genetic screening Raizen, Lee, and Avery (1995) identified *eat-2* (encoding a non-α nAChR subunit) and *eat-18* (encoding a single pass transmembrane domain protein with no vertebrate homologue) as essential components of pharyngeal cholinergic transmission in *Caenorhabditis elegans*. Here, we have cloned and functionally expressed EAT-2 and EAT-18 from free living (*C. elegans)* and parasitic (*Ascaris suum)* nematodes. For the first time we find that a non-α nicotinic subunit (EAT-2) can form a homomeric ligand-gated cation selective ion-channel. The functional expression of this noncanonical receptor is dependent on co-expression with EAT-18. All previously characterized cation selective nAChRs have at least two α subunits with the ligand binding sites located at the interface between each α and its adjacent subunit (Holden-Dye et al. 2013; Corringer, Novère, and Changeux 2000). We have used electrophysiological, biochemical and molecular techniques to demonstrate that EAT-18 forms part of the mature receptor and functions as an obligate auxiliary protein.

3.3 Methods

3.3.1 Molecular Biology

Plasmid constructs (GeneArt®) containing *C. elegans* EAT-2 (Accession number: Y48B6.4) & EAT-18 (Accession number: isoform-c - Y105E8A.7c.1 and isoform-d -Y105E8A.7d.1) were cloned into *XhoI* and *ApaI* restriction sites of the pTB-207 expression vector using In-Fusion cloning kit (Takara Bio USA, Inc.; EAT-2: 5' end - TGGCGGCCG*ctcgag*ATGACCTTGAAAATCGCATTTTTCA and 3' end - ATCAAGCTC*gggccc*TTATTCAATATCAACAATCGGACTAT; EAT-18: 5' end – TGGCGGCCG*ctcgag*ATGCGAAGCCTGGAGCGAAT and 3' end - ATCAAGCTC*gggccc*TCAAAGTGTTGATCGCATTTCCTCA). For biochemistry and immunofluorescence assays, *Cel*-EAT-2 was tagged with GFP in between the transmembrane region 3 and 4 between leucine 377 and 378; EAT-18 was tagged with the 6xHis tag at the C-terminal. Full length sequences of *A. suum* EAT-2 (Accession number: GS_09411) and EAT-18 were amplified from total RNA extracted from dissected whole pharynx of *A. suum*. Briefly, TRIzol Reagent™ (Invitrogen™, Carlsbad, CA, USA) was used to extract total RNA from *A. suum* adult worms. cDNA was synthesized by using SuperScript VILO Master Mix (Invitrogen™, Carlsbad, CA, USA) and served as a template for the amplification. Full length product was sub-cloned into pTB207 expression vector by adding *XhoI* and *ApaI* restriction enzyme sites respectively to the forward primer (5' end: TGGCGGCCG*ctcgag*ATGCAAATATTTTCTATGGTAATT) and reverse primers (3' end: ATCAAGCTC*gggccc*TTAATTCCATACGTTTGGGG) using In-Fusion cloning. Z-competent *E. coli* JM109 cells (Zymo Research, Irvine, CA) were used for transformation

of the ligated product. The final cloned constructs of all the plasmids were sequenced with pTB207 vector primers (forward, T7) and (reverse, SP6). Only positive clones were used for cRNA synthesis using in vitro transcription with the mMessage mMachine T7 transcription kit (Invitrogen, CA, USA) and the cRNA was aliquoted and stored at -80°C.

3.3.2 Electrophysiology

3.3.2.1. Two-electrode voltage-clamp in *Xenopus* oocytes

Oocyte injections and two-electrode electrophysiology recordings were performed as previously described (Choudhary et al. 2019).

3.3.2.2. *A. suum* current clamp recordings from the pharynx

A. suum pharyngeal dissections and electrophysiology recordings were adapted from Martin (Martin 1985). Briefly, pharynx was dissected out from the head region of the worm and mounted on Sylgard™-lined in a double jacketed bath chamber maintained at 28 °C. The muscle layer surrounding the anterior 3rd of the pharynx was preserved for anchoring. The intestine attached to the posterior end of the pharynx was used for stretching and pinning down. The preparation was continuously perfused with calcium free *Ascaris* Perienteric Fluid-Ringer (calcium free APF-Ringer) composition (mM): NaCl 23, Na-acetate 110, KCl 24, $MgCl_2$ 11, glucose 11, and HEPES 5; NaOH or acetic acid was added to adjust the pH to 7.6. The experimental compounds were dissolved in calcium free APF-Ringer and applied as described in the results. The rate of localized perfusion was 3.5–4 ml min^{-1} through a 20-gauge needle which was placed directly above the recording region of pharynx. The pharyngeal preparations with resting membrane potentials less than -15 mV and the resting conductances less than 250 μS were selected for analysis. We used 3 M potassium acetate in our micropipettes to get the

final resistances of 4-7 MΩ for the voltage sensing and 0.5-1 MΩ for the current injecting electrode for current clamp recordings. The current-injecting electrode injected hyperpolarizing step currents of −1000 nA for 500 ms at 0.3 Hz.

3.3.2.3. *Data analysis*

GraphPad Prism 8.0 software (GraphPad software Inc., CA, USA) was used to analyze the data. In two-electrode voltage-clamp recordings, the peak currents were measured and normalized to 100 μM acetylcholine response and expressed as mean ± S.E.M. The data for sigmoid concentration-response curves was fitted to the Hill equation (Boulin et al. 2008). We used One-way analysis of variance (ANOVA) and Extra sum of squares F-test to test statistical differences (statistically different if $p<0.05$). Tukeys multiple comparison was used as *post-hoc* test.

In current-clamp recordings, the peak changes in membrane conductance (δG_{max}) in response to drug applications were normalized to δG response to acetylcholine application (100 μM ACh, applied for 10s) within each preparation. We constructed the sigmoidal concentration response plots by fitting the data by nonlinear regression to determine the pEC_{50} and the maximal response (R_{max}). Extra sum of squares F-test was used to test statistical differences between pEC_{50}, slope and maximal response. The significance levels were set to $P<0.05$.

3.3.3 Biochemistry

3.3.3.1. Immunostaining for confocal microscopy

Oocytes were prepared for confocal imaging following previously published protocol (Bianchi and Driscoll 2006). In brief, oocytes were fixed in 4%

paraformaldehyde at 4°C overnight. Fixed oocytes were embedded in 3% low-melting point agarose and 50-µm thick slices were cut using a vibratome. The oocyte slices were blocked with 0.2% Bovine Serum Albumin (BSA) plus 0.1% Tween 20 in PBS overnight at 4°C. This was followed by incubation with the primary antibody (ABfinity™ Histidine tag recombinant rabbit oligoclonal antibody at 1:500 dilution for detecting *Cel*-EAT-18c, ThermoFisher#A-710286, and goat anti-GFP rabbit IgG antibody for detecting *Cel*-EAT-2, Abcam # ab6673, 1:1000) over night at 4°C and then incubated with a fluorescent secondary antibody (Alexa Fluor® 488 goat anti-rabbit IgG antibody, 1:1000, ThermoFisher#A-11008; Alexa Fluor Plus 680 donkey anti-goat IgG secondary antibody, ThermoFisher#A-32860, 1:15000) for 1 hour at room temperature. The slices were mounted on glass slides using a Fluoromount^{TM} mounting medium followed by confocal imaging (Leica SP5 X MP confocal/multiphoton microscope system).

3.3.3.2. Western blot analysis using *Xenopus* oocytes

Oocyte protein extraction and western blot analysis protocol were adapted from Lin-Moshier & Merchant (Lin-Moshier and Marchant 2013) with the following modifications. Ten oocytes with currents ≥ 500 nA in response to 100 µM acetylcholine were pooled and suspended in 100 µL of homogenization buffer (50 mM HEPES, pH = 7.5, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, pH = 8.0 and Protease Inhibitor cocktail, Sigma Aldrich, MO, USA). The homogenized sample was centrifuged at 800*g* for 5 min at 4°C and the supernatant was transferred into a clean tube. The centrifugation step was repeated twice to ensure complete removal of the yolk particles. Samples were boiled with Laemmli buffer for 5 min and then subjected to electrophoresis (4-12% Bis Tris gel for *Cel*-EAT-2-GFP and 16% Tricine gels for *Cel*-EAT-18-His). The gels were blotted

onto PVDF membranes and blocked with BlockOut® blocking buffer (Rockland immunochemicals Inc., PA, USA). The blots were probed with a 1:10000 dilution of primary antibody (mouse monoclonal anti-GFP antibody to detect GFP tagged *Cel*-EAT-2, Proteintech # 66002-1-Ig; HRP-conjugated anti-His antibody to detect His tagged *Cel*-EAT-18, Proteintech # HRP-66005) at 4°C overnight. HRP conjugated anti-mouse antibody at 1:10000 dilution was used as secondary antibody (SA00001-1) for GFP tagged protein. Immunoreactivity was visualized by enhanced chemiluminescence (GE healthcare, IL, USA).

3.3.3.3. Co-immunoprecipitation using *Xenopus oocytes* membrane extracts

Xenopus laevis oocytes were processed as described previously; anti-GFP-Trap-A beads (ChromoTek, Germany) were used for immunoprecipitation (Boulin et al. 2012). Laemmli buffer was used to recover the immunoprecipitates and eluates were analyzed separately using the following primary antibodies: mouse monoclonal anti-GFP antibody (Proteintech # 66002-1-Ig, 1:10000), HRP-conjugated anti-His antibody (Proteintech # HRP-66005). HRP conjugated anti-mouse antibody was used as secondary antibody (1:10000; SA00001-1). Chemiluminescent reagents (GE healthcare, IL, USA) were used for detection.

3.4 Results

3.4.1 *Cel*-EAT-2 forms a functional homomeric receptor when co-expressed with *Cel*-EAT-18

Initiation of the pharyngeal muscle action potential and the frequency of excitatory pharyngeal pumping are under the control of the marginal cell (MC) motor neuron in *C. elegans*. MC neurotransmission requires acetylcholine and the nAChR

subunit *Cel*-EAT-*2* which is expressed in pharyngeal muscle (Avery and Horvitz 1990; Raizen, Lee, and Avery 1995). In order to reconstitute the post-synaptic pharyngeal nAChR, we expressed *Cel*-EAT-2 in *Xenopus laevis* oocytes but failed to observe electrophysiological evidence for the formation of a functional nAChR. This could be attributed to the lack of vicinal cysteines required for agonist binding and pointed to a possible requirement for an additional protein or subunit. Raizen, Lee, and Avery (1995) have shown that similar to *Cel-eat-2,* mutations in *Cel-eat-18* rendered worms incapable of MC neurotransmission and rapid pharyngeal pumping suggesting the protein may act as either an ancillary or auxiliary protein for the assembly of a functional nAChR. *Cel-eat-18* is a small, single-pass transmembrane protein. There are two splice variants of the protein in *C. elegans*, EAT-18c (71 aa) and EAT-18d (78 aa), which differ mainly in the C-terminal region (Supporting Information S3.0). We co-expressed *Cel*-EAT-2 with *Cel*-EAT-18c or *Cel*-EAT-18d cRNA and recorded robust responses to 100 μM acetylcholine in both cases (Figure 3.0A). The resulting nAChRs produced larger current amplitudes in response to acetylcholine application when the *Cel*-EAT-18c isoform was used. The majority of subsequent recordings were done using the *Cel*-EAT-18c isoform. The ability of the non-α EAT-2 to express functionally when co-injected with a non-subunit protein makes this cation selective nAChR unique to date.

3.4.2 *Cel*-EAT-2 is a non-α nAChR subunit most similar to vertebrate α-7 subunits

C. elegans EAT-2 has the typical functional domains of a pentameric ligand-gated ion channel subunit: a large extracellular N-terminal domain of ~200 amino acids required for correct nAChR assembly and agonist binding; a cys-loop separated by 13

intervening amino acids; four transmembrane (TM) domains that form the ion-conducting pore; a cytoplasmic domain between TM3 and TM4 that is involved in modulation of channel activity and ion conductance; and a short extracellular C-terminus. EAT-2 is a non-α subunit as it lacks the pair of adjacent cysteine residues in loop-C required for agonist binding, but overall its sequence is most comparable to the human α-7 subunit with 55% similarity in amino acid residues (Supporting Information S3.1). Ligand binding occurs in a cleft formed by three loops (A, B, C) of the principal face of one α subunit and a series of beta strands from loops (D, E, F) of the complimentary interface of the adjacent subunit. All α subunits have either a YXCC or YXXCC motif in loop-C and this motif was considered essential for ligand binding and modulating the affinity of the receptor binding site (Kao et al. 1984; Karlin 2002; Kao and Karlin 1986). EAT-2 lacks the YXCC or YXXCC motif but is still able to assemble, with EAT-18, as a functional cation selective homomeric channel, which encouraged us to continue to explore this interesting and different nAChR. This lack of vicinal cysteines in the EAT-2 protein subunit suggest that the receptor channel will have different contact residues in the ligand binding pocket and a different pharmacology from other nAChRs.

3.4.3 Pharmacology of the *Cel*-EAT-2 nAChR

In order to investigate the potential of EAT-2 as a drug target, we characterized the pharmacology of the nAChR by using two-electrode voltage-clamp. Different cholinergic agonists and anthelmintic agents were tested on the heterologously expressed *Cel*-EAT-2 receptor. All agonists were used at 100 μM, except tribendimidine which was tested at 30 μM ($n \geq 6$ for all agonists). Methacholine was the most efficacious cholinergic agonist (I_{max} = 73 ± 5.3%) after acetylcholine followed by nicotine (I_{max} = 55

± 8.0%). Oxantel acted as a weak agonist and produced 11 ± 1.3% of the control acetylcholine response. However, many of the current cholinergic anthelmintic drugs such as morantel, levamisole, bephenium, tribendimidine and pyrantel did not activate the receptor. Fig. 3.0B shows the rank order potency series for agonists and anthelmintics on the *Cel*-EAT-2 receptor when normalized to control 100 μM acetylcholine (ACh) current response: acetylcholine > methacholine > nicotine > carbachol > butyrylcholine > epibatidine > oxantel >>> DMPP (Dimethylphenylpiperazinium) = tribendimidine = bephenium = cytisine = lobeline = levamisole = SIB 1508Y = α-cotinine = nornicotine = anabasine = pyrantel.

To further investigate the receptor pharmacology, we examined the concentration-response relationships of selected agonists (Figure 3.0 C&D and Supporting Information S3.2A). 100 μM ACh was used as the internal standard for normalization. Nicotine (pEC_{50} = 4.2 ± 0.1) was the most potent agonist after acetylcholine (pEC_{50} = 4.8 ± 0.0), whereas carbachol was least potent with a pEC_{50} = 3.4 ± 0.0. The concentration-response curves for all the agonists had hill coefficient values greater than 1 indicating positive cooperativity, with methacholine having the steepest hill slope (n_H = 3.5 ± 1.3). This suggests that the *Cel*-EAT-2 ion channel has multiple ligand binding sites consistent with other nAChRs.

To characterize the antagonist pharmacology, we tested the effects of five cholinergic antagonists on the expressed *Cel*-EAT-2 channel. The antagonists were α-bungarotoxin (10 μM), derquantel (10 μM), paraherquamide (30 μM), d-tubocurarine (30 μM) and dihydro-β-erythroidine (30 μM, DHβE). Figure 3.0 E&F (and Supporting Information S3.2B) illustrate the effect of various antagonists on the acetylcholine

concentration-response relationship for *Cel*-EAT-2. d-Tubocurarine produced the most potent inhibition and almost completely blocked the response mediated by acetylcholine (≈98% inhibition). Unlike many mammalian nAChRs, sensitivity and efficacy of the receptor for acetylcholine was not altered by either α-bungarotoxin or DHβE. The rank order potency series based on mean current (%) decrease of the control 100 μM ACh current response was: d-tubocurarine > paraherquamide > derquantel >>> *α*-bungarotoxin ≈ DhβE. In conclusion, the pharmacology of the *Cel*-EAT-2 receptor is distinct from previously characterized nematode and vertebrate nAChRs (Buxton et al. 2014; Abongwa, Baber, et al. 2016; Ballivet et al. 1996; Raymond, Mongan, and Sattelle 2000; Chen and Patrick 1997; Decker et al. 1995).

3.4.4 Characterization of the acetylcholine response in the *A. suum* pharynx

Although *C. elegans* is a powerful model, it is not a parasitic nematode of medical importance. In order to validate pharyngeal nicotinic acetylcholine ion channels as potential anthelmintic drug targets, it is crucial to identify and establish the presence and in turn the pharmacology of such nAChRs in the pharynx of parasitic worms. We therefore characterized the pharmacology of the *A. suum* pharynx for comparison with the *Cel*-EAT-2 receptor. We employed the current-clamp technique to understand the pharmacology of the postsynaptic nAChR response. Application of 100 μM acetylcholine on the pharyngeal preparation produced a large depolarization accompanied by an increase in membrane conductance. The acetylcholine response was inhibited by mecamylamine and the preparation showed negligible responses to several muscarinic

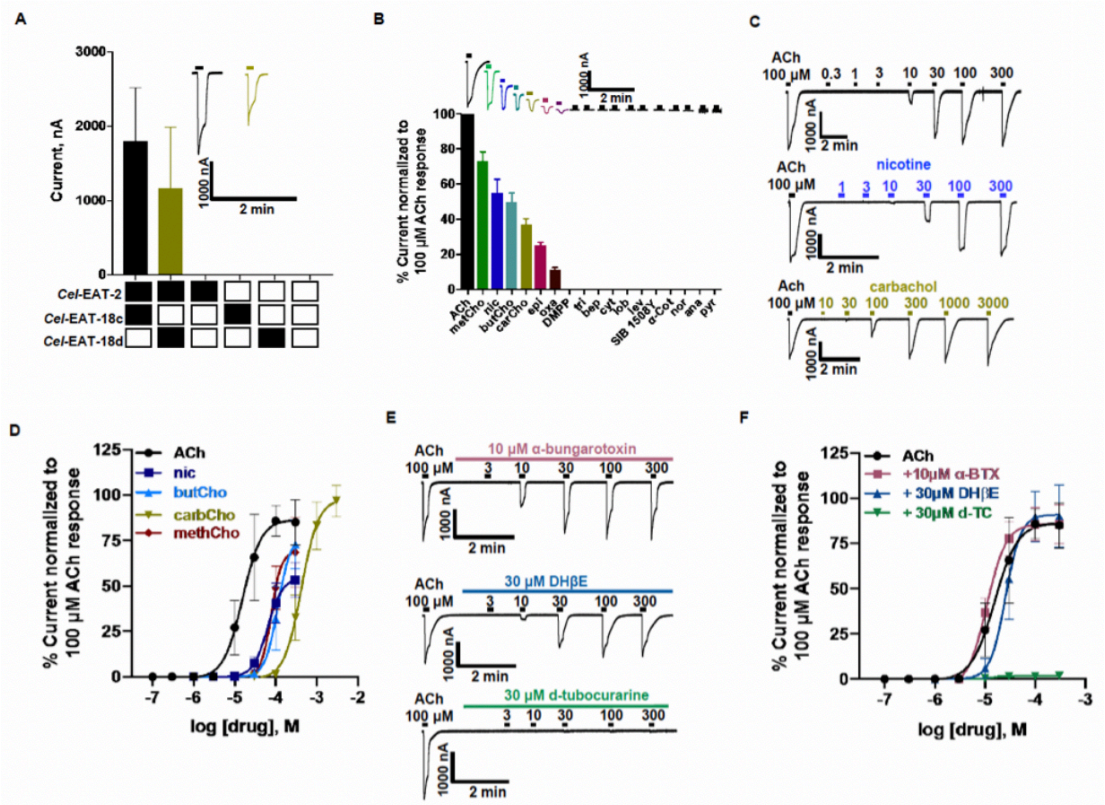

Figure 3.0 Pharmacology of the *Cel*-EAT-2 nicotinic acetylcholine receptor. A. Bar graph showing current sizes (mean±S.E.M, %) produced in response to 100 µM acetylcholine for various mixtures of *Cel*-EAT-18c & d and *Cel*-EAT-2. B. Bar graph (expressed as mean±SEM, %, n≥6) showing rank order potency for nAChR agonists and anthelmintics when normalized to the control 100 µM ACh current: acetylcholine (ACh) > methacholine (methCho; 73.0±5.3) > nicotine (nic; 55.0±8.0) > butyrylcholine (butCho; 50.0±5.0) > carbachol (carCho; 37.0±3.4) > epibatidine (epi; 25.0±1.5) > oxantel (oxa; 11.0±1.3) >>> dimethylphenylpiperazine (DMPP; 0.0±0.0) = tribendimidine (tri; 0.0±0.0) = bephenium (bep; 0.0±0.0) = cytisine (cyt; 0.0±0.0) = lobeline (lob; 0.0±0.0) = levamisole (lev; 0.0±0.0) = SIB 1508Y (0.0±0.0) = α-cotinine (α-cot; 0.0±0.0)= nornicotine (nor; 0.0±0.0) = anabasine (ana; 0.0±0.0) = pyrantel (pyr; 0.0±0.0). C. Sample traces for acetylcholine, nicotine and carbachol concentration–response relationships. D. Concentration-response plots of selected agonists (n≥6). pEC_{50} (mean±SEM) and hill slope (n_H, mean±SEM) values were respectively: 4.8±0.0 and 1.9±0.3 for ACh; 4.2±0.1 and 2.4±0.4 for nic; 4.1±0.0 and 3.5±1.3 for methCho, 3.9±0.1 and 2.8±1.8 for butCho; 3.4±0.0 and 2.1±0.3 for carbCho. E. Sample traces for acetylcholine concentration–response relationships in the presence of 10 µM α-bungarotoxin (α-BTX), 30 µM DHβE (Dihydro-β-erythroidine) and 30 µM d-tubocurarine (d-TC). F. Acetylcholine concentration-response curves in the presence of α-BTX (n=7), DHβE (n=6) and d-TC (n=6). d-TC caused ≈98% reduction in the mean acetylcholine response. α-BTX (pEC_{50} = 5.0±0.0 and I_{max} = 86.0±2.4%) and DHβE (pEC_{50} = 4.6±0.0 µM and I_{max} = 91.1±4.1%) failed to show any significant antagonistic effects on the response mediated by acetylcholine.

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agonists (Supporting Information Data S3.0). This confirmed the presence of a nicotinic acetylcholine receptor (nAChR) in the pharynx of the parasite.

We next quantified the effects of selected nicotinic agonists to determine whether the pharyngeal nAChRs are pharmacologically distinct from those of somatic muscle. Our pharyngeal preparations in this group had a mean resting membrane potential of -21.3 ± 1.3 mV and a mean resting conductance (G) of 136.4 ± 14.9 μS (n=17). The change in conductance (δG) responses to test applications of selected nicotinic agonists were normalized to the ACh δG. Nicotine was the most potent agonist after acetylcholine with mean δG of 92.0 ± 6.2%. Cytisine also produced a large conductance change in the *A. suum* pharynx (mean δG = 71.2 ± 5.0). The rank order potency series for vertebrate nicotinic agonists on the *A. suum* pharynx was: ACh > nicotine > cytisine > epibatidine > DMPP >> choline (Figure 3.1A). The rank order potency series of selected vertebrate nicotinic agonists on the pharynx differs from that of somatic muscle nAChRs and vertebrate host nAChRs (Supporting Information Table S3.0). We also tested nine cholinergic anthelmintics on the receptor to study their effect. Our pharyngeal preparations in these experiments had a mean resting membrane potential of -19.3 ± 1.1 mV and a mean resting conductance of 150.5 ± 11.9 μS (n=21). The δG responses to test applications of selected cholinergic anthelmintic agents were normalized to the ACh δG. Figure 3.1B shows the rank order potency series on the *A. suum* pharynx: ACh >> bephenium > thenium > levamisole ≈ morantel ≈ pyrantel ≈ oxantel ≈ methyridine ≈ tribendimidine. In contrast to somatic muscle nAChRs, none of the cholinergic anthelmintics tested on the pharynx produced >7% of the acetylcholine response (Supporting Information Table S3.0).

To further investigate the receptor, we used selected nicotinic antagonists (30μM) to study their inhibitory effects on 100μM ACh responses. Our pharyngeal preparations in this group had a mean resting membrane potential of -20.2 ± 1.1 mV and a mean resting conductance of 129.2 ± 6.8 μS (n=34). The δG produced by a control application of ACh was set as 100%. We calculated the % inhibition of the δG response to ACh by nicotinic antagonists to determine a rank order potency series (mean ± SEM, Supporting Information S3.3): d-tubocurarine > mecamylamine > methyllycaconitine > paraherquamide > derquantel > hexamethonium > DHβE. The rank order potency series of nicotinic receptor antagonists on the pharynx differs from that of vertebrate nAChRs (Supporting Information Table S3.0).

We also determined concentration response curves by plotting the concentration of agonists (1-1000μM, applied for 10s) against the response (δG) produced. The δG produced during the increasing concentration of agonist was normalized to the δG produced by 100 μM ACh (applied for 10s) within each experiment. Figure 3.1C shows the concentration response curves for ACh and nicotine. The pEC_{50} of ACh and nicotine were 5.0 ± 0.0 and 5.0 ± 0.1. The maximal response of ACh was 105.3 ± 2.5 μS and nicotine was 79.3 ± 4.3 μS. We determined the ACh concentration-response relationships in the presence of nicotinic receptor antagonists: d-tubocurarine (10μM), methyllycaconitine (10μM), paraherquamide (10μM) and dihydro-β-erythroidine (30μM) (Figure 3.1D). The pEC_{50} of the ACh concentration response curve did not significantly differ in the presence of methyllycaconitine, dihydro-β-erythroidine, d-tubocurarine or paraherquamide but the maximal response for ACh was inhibited. This suggests that these compounds act as non-competitive antagonists of the pharyngeal ACh response.

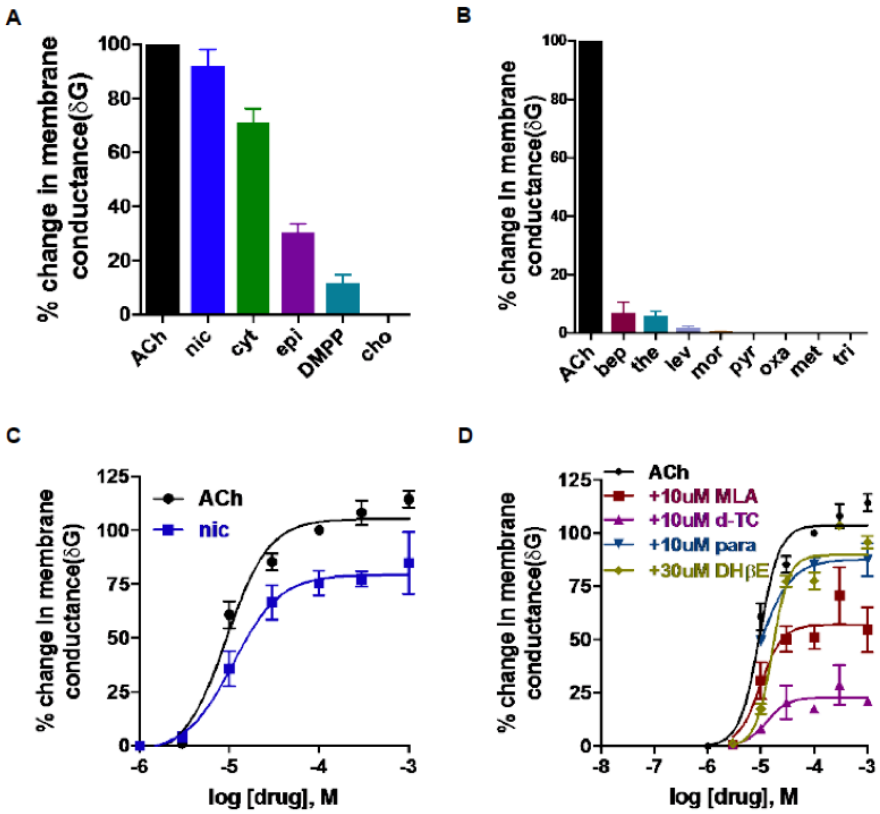

Figure 3.1 Pharmacology of the *A. suum* pharynx. A. Bar graph showing the rank order potency of selected vertebrate nAChR agonists producing % change in membrane conductance (δG; expressed as mean±SEM,%, n≥4): ACh (100.0±0.0) > nicotine (nic; 92.0±6.2) > cytisine (cyt; 71.0±5.0) > epibatidine (epi; 31.0±3.0) > dimethylphenylpiperazine (DMPP; 12.0±2.9) >> choline (cho; 0.0±0.0). B. Bar graph showing the rank order potency series of selected cholinergic anthelmintics producing % change in membrane conductance (δG; expressed as mean±SEM,%)*:* bephenium (bep; 7.2±3.5) > thenium (the; 6.1±1.5) > levamisole (lev; 1.8±0.61) >>> morantel (mor; 0.3±0.3) > pyrantel (pyr; 0.0±0.0) = oxantel (oxa; 0.0±0.0) = methyridine (met; 0.0±0.0) = tribendimidine (tri; 0.0±0.0). C. Concentration-conductance curves for ACh and nicotine plotting % change in conductance vs log molar concentration of the drugs. pEC_{50} (mean±SEM) and hill slope (n_H, mean±SEM) values were respectively: 5.0±0.0 and 1.8±0.3 for acetylcholine (n=6) and 5.0±0.1 and 1.7±0.6 for nicotine (n=8). D. Concentration-conductance plots of ACh in the presence of nAChR antagonists: paraherquamide (para; 10μM), methyllycaconitine (MLA; 10μM), d-tubocurarine (d-TC; 10μM) and Dihydro-β-erythroidine (DHβE; 30μM). The pEC_{50} values were 5.0±0.1 in the presence of MLA (n = 8); 4.9±0.2 in the presence of d-TC (n = 3); 5.1±0.1 in the presence of para (n = 3) and 4.8±0.0 in the presence of DHβE (n = 7). The maximal response (δG) (mean±SE, μS) values were: 57.0±4.6 in the presence of MLA; 23.0±3.0 in the presence of d-TC; 87.7±5.7 in the presence of para; 89.7±3.0 in the presence of DHβE.

3.4.5 Functional expression of *Asu*-EAT-2 requires *Asu*-EAT-18 and *Asu*-RIC-3

After characterizing the *A. suum* pharyngeal nicotinic response, we were interested in identifying the subunits which constitute these ion channels. We used *Cel*-EAT-2 and *Cel*-EAT-18 sequences as queries in BLASTP homology searches and identified homologues for EAT-2 and EAT-18 in the pig parasite. Comparison of *Asu*-EAT-2 with *Cel*-EAT-2 sequences revealed 80% similarity in amino acid composition, with differences among some of the ligand binding residues from various loops (Supporting Information S3.1). This suggested that the receptor channel could have different contact residues in the ligand binding pocket and possibly a different pharmacology. The proteins were expressed *in vitro* in *Xenopus* oocytes to recapitulate the pharyngeal ligand-gated cation channel. Unlike *Cel*-EAT-2 nAChRs, *Asu*-EAT-2 not only required *Asu*-EAT-18 but also *Asu*-RIC-3 for robust expression. However, addition of *Asu*-UNC-50 or *Asu*-UNC-74 did not produce any significant increase in current amplitude. The most robust responses were observed from oocytes injected with 30 ng each of *Asu*-EAT-2 and *Asu*-EAT-18 plus 20 ng of *Asu*-RIC-3 (Supporting Information S3.4), and so these cRNA amounts were used for all subsequent injections.

3.4.6 Pharmacological profile of the *Asu*-EAT-2 nAChR

To determine the agonist pharmacology of the *Asu*-EAT-2 receptor we tested various cholinergic agonists and anthelmintic agents. The rank order potency series based on maximum current response (Figure 3.2A) for the receptor was: nicotine > acetylcholine > cytisine > epibatidine > DMPP > oxantel. As with the *A. suum* pharynx, cholinomimetic anthelmintics such as bephenium, tribendimidine, levamisole and pyrantel failed to

activate the receptor. We also constructed a concentration-response curve for acetylcholine and found it to be ≈ 9 times more potent on the *Asu*-EAT-2 nAChR compared to *Cel*-EAT-2 with a $pEC_{50} = 5.7 \pm 0.0$ (Figure 3.2B). We tested the antagonistic effects of derquantel, mecamylamine, d-tubocurarine, DHβE, hexamethonium and methyllycaconitine on the *Asu*-EAT-2 receptor. The mean % inhibition of the 100 μM acetylcholine current response was used to determine the rank order potency of the antagonists. Mecamylamine and d-tubocurarine produced almost 100% inhibition of the acetylcholine currents and DhβE was the least potent antagonist (inhibition, 66 ± 8.4%). The rank order potency series for the antagonists (Figure 3.2C) was: d-tubocurarine ~ mecamylamine > hexamethonium > methyllycaconitine > deraquantel > DhβE. The rank order potency series of cholinomimetic anthelmintics, nicotinic agonists and antagonists on the *Asu*-EAT-2 receptor differs from that of the *A. suum* somatic muscle nAChRs as well as the vertebrate nAChRs (Supplementary Information S3.0). In conclusion, the *Asu*-EAT-2 receptor has a distinct pharmacology and is therefore likely suitable to be exploited as a therapeutic target.

3.4.7 Tissue expression of *eat-2* and *eat-18* in *A. suum*

In *C. elegans,* EAT-2 expression is restricted to pharyngeal muscle, while EAT-18 is found in both pharyngeal muscle and some neurons (McKay et al. 2004). We used RT-PCR to examine the distribution of *Asu-eat-2* and *Asu-eat-18* mRNA in various dissected adult *A. suum* tissues and single somatic muscle cells (n≥ 5; Figure 3.2D). We determined that *Asu-eat-2* was transcribed in the pharynx, sections of the reproductive tract and the head region. RT-PCR results revealed the presence of *Asu-eat-18* message in

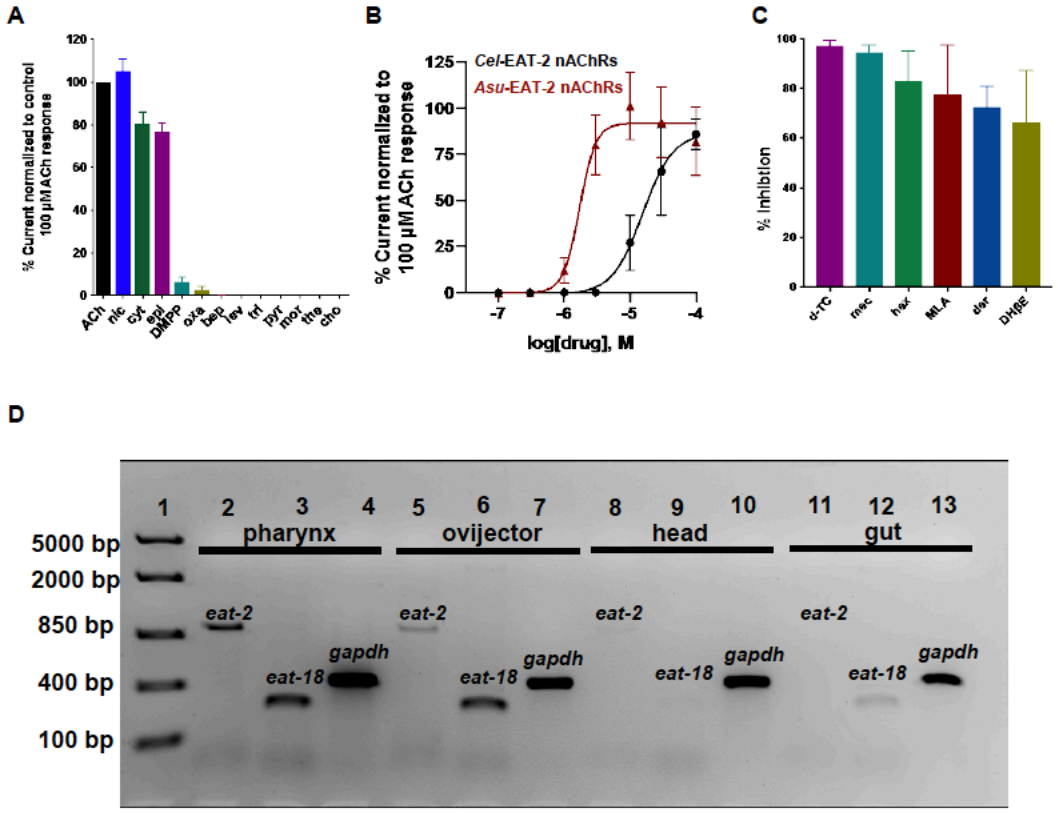

Figure 3.2: Effect of selected cholinergic agonists, anthelmintics and antagonists on the *Asu*-EAT-2 receptor. A. Bar graph showing the rank order potency (mean ± SEM, %, n≥5) of cholinergic agonists and anthelmintics when normalized to the control 100 µM ACh current: nicotine (nic; 105.0±5.7) ≈ acetylcholine (ACh; 100±0.0, n=9) > cytisine (cyt; 81.0±5.2) > epibatidine (epi; 77.0±4.2) > dimethylphenylpiperazinium (DMPP; 6.6±1.9) > oxantel (oxa; 3.0±1.3) >>> bephenium (bep; 0.1±0.1, n=9) = tribendimidine (tri; 0.0±0.0) = levamisole (lev; 0.0±0.0) = pyrantel (pyr; 0.0±0.0) = choline (cho; 0.0±0.0) = thenium (the; 0.0±0.0). B. Comparison of concentration-response plots to acetylcholine for the *Cel*-EAT-2 (black curve) and *Asu*-EAT-2 (maroon curve) receptor. pEC_{50} (mean ± SEM) and hill slope (n_H, mean ± SEM) values were respectively: 4.8 ± 0.0 and 1.9 ± 0.2 for *Cel*-EAT-2 (n=9); 5.8 ± 0.1 and 3.5 ± 1.1 for *Asu*-EAT-2 (n=6). C. Bar graph showing the rank order potency of selected vertebrate nAChR antagonists. Results were expressed as mean ± SEM (%, n=6), inhibition of currents elicited by 100 µM ACh. d-Tubocurarine (d-TC, 10µM; 97±1.0) and mecamylamine (mec, 10µM; 95±1.1) almost completely blocked the acetylcholine response. Mean current inhibition were 83.0±5.1 for hexamethonium (hex, 10µM), 78.0±8.1 for methyllycaconitine (MLA, 10µM), 72.0±3.4 for derquantel and 66±8.4 for DHβE (30µM, Dihydro-β-erythroidine). D. Localization of *Asu-eat-2* and *Asu-eat-18* mRNA in different body tissues of the *A. suum* worm (n = 5). RT-PCR analysis of *Asu-eat-2* (lanes 2, 5, 8,11) and *Asu-eat-18* (lanes 3, 9, 9,12) and *gapdh* control (lanes 4, 7, 10, 13) in pharynx, ovijector, head, and gut region. The PCR product sizes for *eat-2, eat-18* and *gapdh* were 949, 213 and 411 bp respectively. Lane 1, FastRuler High Range DNA ladder.

the same tissues, as well as gut tissue. We found no evidence of expression of *Asu-eat-2* or *Asu-eat-18* in somatic muscle cells (Supplementary Information S3.5). The widespread expression of both the proteins in body tissues other than pharynx was unexpected. It is plausible that *Asu*-EAT-2 not only assists in feeding but also plays a role in other physiological processes such as reproduction. It also raises the possibility that EAT-18 is interacting with other nAChR subunits in different tissues.

3.4.8 Comparative pharmacological profile reveals EAT-2 constitutes the pharyngeal nAChR in *A. suum*

Figure 3.3A shows the pharmacological comparison between *in vitro Cel*-EAT-2, *Asu*-EAT-2 and *in vivo A. suum* pharyngeal recordings. The agonist rank order potency series acquired from both *in vivo* and *in vitro* recordings in *A. suum* revealed a similar pharmacological profile. Both nicotine and cytisine were highly efficacious in *in vivo* and *in vitro* recordings in *A. suum,* while DMPP acted as a weak agonist. In comparison, the *Cel*-EAT-2 channel failed to respond to cytisine and DMPP application but was activated by oxantel (I_{max} = 11.0 ± 1.3 % of acetylcholine response) albeit weakly. Importantly, the comparable pharmacological profile observed for *Ascaris in vivo* and *in vitro* recordings suggests it is likely that EAT-2 and EAT-18 constitute the pharyngeal nicotinic response in the parasitic nematode.

3.4.9 Different EAT-18 homologues affect the pharmacology of the EAT-2 nAChR

We expected to see differences in nAChR pharmacology between *A. suum* and *C. elegans* due to differences in the amino acid residues of the EAT-2 protein sequences. EAT-2 cannot form a functional receptor on its own and requires EAT-18. To determine

the pharmacological relevance of EAT-18, we expressed *Cel*-EAT-2 with *Asu*-EAT-18. We tested five agonists on the expressed channel: acetylcholine, nicotine, cytisine, levamisole, tribendimidine and pyrantel (data not shown). No significant differences were observed in the rank order potency series. Interestingly, the nicotine sensitivity was affected illustrating a change in the pharmacology (Figure 3.3B). Substitution of *Asu*-EAT-18 for *Cel*-EAT-18c shifted the concentration-response curve to the left and increased the efficacy of nicotine on the receptor. The EC_{50} = 18.7 μM (pEC_{50} = 4.7 ± 0.0) for nicotine was approximately 3.5 times lower than before 64.2 μM (pEC_{50} = 4.2 ± 0.0). We also observed a significant increase in I_{max} (96.5 ± 2.3% from 54.6 ± 2.6%) when replacing *Cel*-EAT-18c with *Asu*-EAT-18. Sensitivity of the pharyngeal receptor to acetylcholine was also altered significantly but there was no effect on the agonist efficacy (Supplementary Information S3.6A and B). The significant shift in acetylcholine and nicotine pEC_{50} establishes the electrophysiological evidence of modulation of the receptor by EAT-18 and points to an interaction between the proteins.

3.4.10 EAT-18 colocalizes with EAT-2 on the oocyte surface membrane

EAT-18 is required for functional *in vitro* expression of EAT-2 and modulates its pharmacological properties. McKay et al. (2004) have shown that *Cel*-EAT-18 is not required for trafficking of *Cel*-EAT-2 to the oocyte membrane. It is possible that *Cel*-EAT-18 functions as an auxiliary subunit rather than an ancillary chaperone protein. To test this hypothesis, we performed confocal imaging experiments on oocytes expressing GFP tagged *Cel*-EAT-2 and His tagged *Cel*-EAT-18c constructs. These experiments revealed that both *Cel*-EAT-2 and *Cel*-EAT-18c were co-localized on the oocyte surface membrane. Fig. 3.4 C&D shows the images for double immunostained sections of

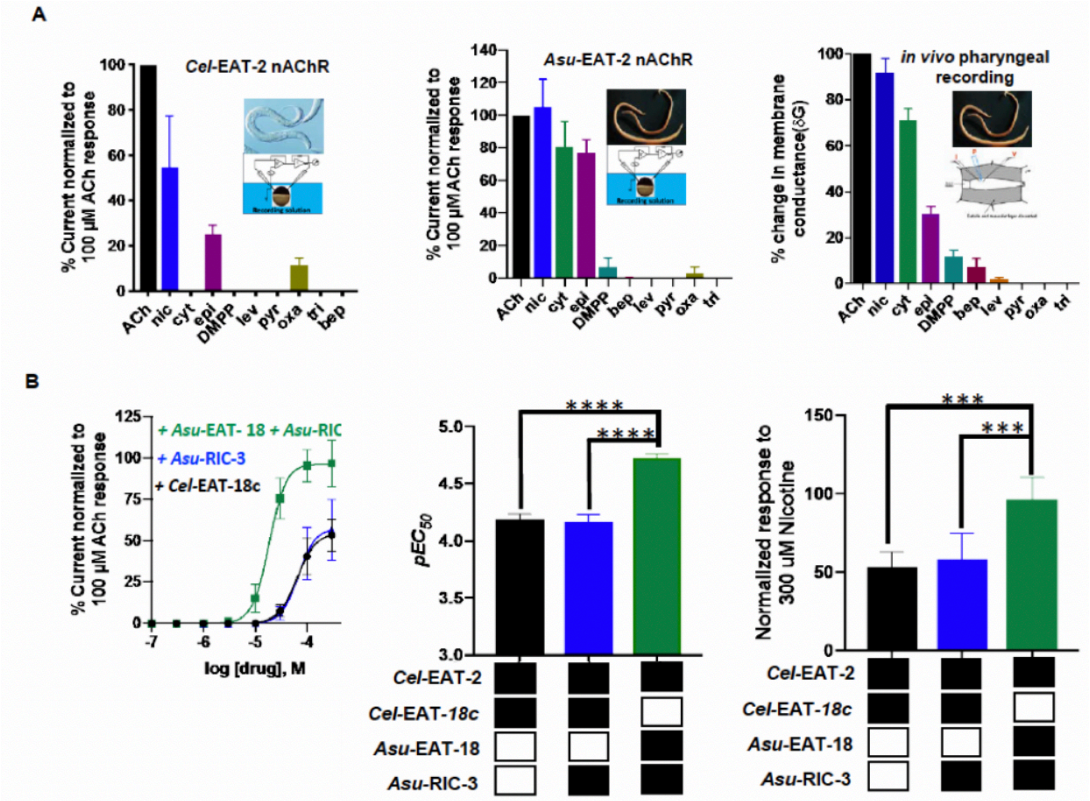

Figure 3.3 A. Bar graph showing comparative pharmacology of agonists for *Cel*-EAT-2 receptor expressed *in vitro*, *Asu*-EAT-2 receptor expressed *in vitro* and *in vivo* pharyngeal recording in *A. suum.* Inset: Images of source nematode (*C. elegans* and *A. suum*) and corresponding recording techniques (TEVC recordings from *X. laevis* oocytes and current-clamp recordings from intact *A. suum* pharynx). B. Effect of different EAT-18 homologues on pharmacology of the *Cel*-EAT-2 receptor. Concentration-response curves showing comparison for nicotine application on *Cel*-EAT-2 + *Cel*-EAT-18c mix (black curve), *Cel*-EAT-2 + *Cel*-EAT-18c + *Asu*-RIC-3 (blue curve) and *Cel*-EAT-2 + *Asu*-EAT-18 + *Asu*-RIC-3 mix (green curve). Bar graphs showing significant effect of using different EAT-18 proteins with *Cel-eat-2* on pEC_{50} (top graph) and on maximum response (bottom graph) produced by application of 300 µM nicotine. $^{***}P < 0.001$, $^{****}P < 0.0001$; significantly different as indicated; Tukey's multiple comparison tests.

injected and un-injected oocytes. *Cel*-EAT-18c is not a typical nAChR subunit protein and its co-localization with *Cel*-EAT-2 suggests the possibility of an association between both proteins. *Cel*-EAT-2-GFP, when expressed alone, localizes to the oocyte surface membrane suggesting that *Cel*-EAT-18c does not function as an ancillary protein (Figure 3.4A). We further assessed the expression of the *Cel*-EAT-2 channel by western blot

analysis of oocyte protein extracts. Using antibodies that recognize GFP and His tags, we detected *Cel*-EAT-2-GFP as a 62 kDa and *Cel*-EAT-18c-His as a 10kDa protein (Figure 3.4E and Supplementary Information S3.9A). We were able to detect *Cel*-EAT-2 in membrane extracts prepared from oocytes co-injected with *Cel*-EAT-2 and *Cel*-EAT-18c as well as oocytes injected with *Cel*-EAT-2 alone. In contrast, *Cel*-EAT-18c was only present in membrane extracts prepared from oocytes co-injected with both *Cel*-EAT-2 and *Cel*-EAT- 18c. We did detect *Cel*-EAT-18c in whole oocyte extracts when *Cel*-EAT-18c was injected alone. It is plausible that EAT-18 requires EAT-2 for trafficking to the surface membrane and its role is more complex than a simple ancillary protein; perhaps related to the functionality of the mature receptor.

3.4.11 EAT-18 forms a part of the EAT-2 nAChR complex

Although *Cel*-EAT-18c was co-localized with *Cel*-EAT-2 on the surface membrane of the oocytes and modulated the pharmacology of pharyngeal nAChR, it did not define the molecular interaction. Therefore, we performed co-immunoprecipitation experiments in order to explore a possible, direct interaction between *Cel*-EAT-18c and *Cel*-EAT-2 (Figure 3.4F and Supplementary Information S3.9B). We were able to demonstrate that *Cel*-EAT-18c-His co-immunoprecipitated with *Cel*-EAT-2-GFP, which shows that EAT-18 directly interacts with EAT-2 and is a part of the mature receptor complex (Figure 3.4G).

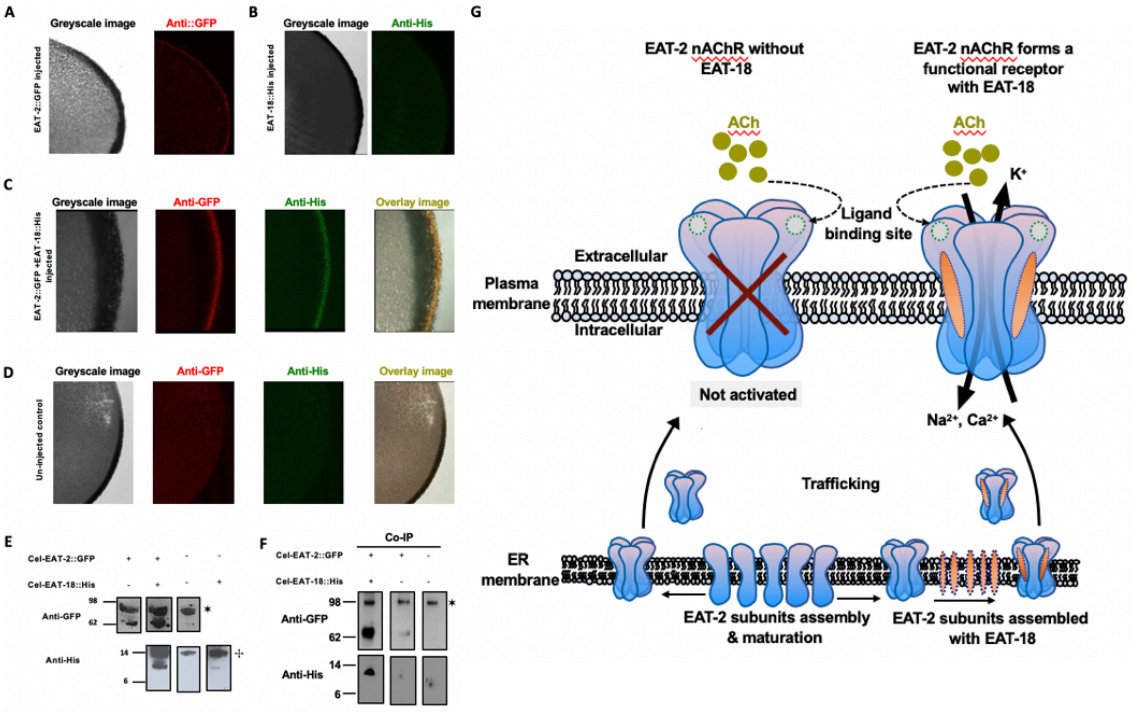

Figure 3.4 EAT-2 and EAT-18 form a receptor complex. A. Immunostained oocyte sections showing expression of *Cel*-EAT-2-GFP (red fluorescence; n=4) on the surface membrane when injected alone. B. *Cel*-EAT-18-His (n=4) fails to localize on the surface membrane when injected alone. C. Double immunostained sections of *Xenopus laevis* oocytes showing *Cel*-EAT-2-GFP and *Cel*-EAT-18-His (n=6) on the surface membrane. The overlay image (yellow fluorescence) shows the co-localization of both the proteins. D. Double immunostained sections of un-injected (negative control; n=6) *Xenopus laevis* oocytes. E. Western blot analysis of *Xenopus* oocyte extracts. Un-injected oocytes served as negative control. *Cel*-EAT-2-GFP was immunostained with anti-GFP antibodies and was present in the extracts prepared from oocytes co-injected with *Cel*-EAT-18-His as well as oocytes injected with *Cel*-EAT-2-GFP alone. *Cel*-EAT-18-His was immunostained with anti-His antibodies and was present in the extracts prepared from oocytes co-injected with *Cel*-EAT-2-GFP. ✶, ✢: non-specific interacting protein bands labelled by anti-GFP and anti-his antibodies respectively, served as positive control. F. Co-immunoprecipitation experiments revealed *Cel*-EAT-18 directly interacts with EAT-2 and constitutes part of the receptor complex. *Cel*-EAT-2-GFP was immunoprecipitated using anti-GFP followed by western blot analysis of *Cel*-EAT-18-His using anti-His antibodies. Un-injected oocytes and oocytes injected with *Cel*-EAT-2-GFP alone served as negative controls for co-immunoprecipitation experiments. ✶: non-specific interacting protein bands labelled by anti-GFP and anti-his antibodies respectively, served as positive control. G. Schematic representation for physical interaction between EAT-2 and EAT-18.

3.5 Discussion

nAChRs are key components of the metazoan neuromuscular junction and important targets for anti-parasitic interventions. They typically are composed of 5 subunits including at least 2 α subunits. Here we describe for the first time a non-α nAChR subunit can form a functional homomeric cation selective receptor. *Cel*-EAT-18 functions as an obligate auxiliary protein and modifies the pharmacological properties of this cys-loop ion channel. A number of previously characterized nAChRs require ancillary proteins, either RIC-3 alone or in combination with UNC-50 and UNC-74, for successful *in vitro* expression (Abongwa, Buxton, et al. 2016; Boulin et al. 2008; Millar 2008; Haugstetter, Blicher, and Ellgaard 2005; Buxton et al. 2014). The *Cel*-EAT-2 receptor instead only required co-expression with EAT-18, which has no similarity to other ancillary proteins, for *in vitro* expression. Both proteins are localized in the pharyngeal muscles and mutations in *Cel-eat-18* caused pharyngeal pumping defects similar to *Cel-eat-2* mutations (McKay et al. 2004; Raizen, Lee, and Avery 1995). Based on our confocal imaging studies, *Cel*-EAT-18c is localized on the oocyte surface only when injected with *Cel*-EAT-2. This supports our hypothesis that EAT-18 interacts with EAT-2 and likely requires the pore forming subunit protein for trafficking to the surface membrane. Other evidence of interaction is provided by the pharmacological modulation of the acetylcholine and nicotine responses by using EAT-18 proteins from different nematode species with *Cel*-EAT-2. The co-immunoprecipitation experiments have provided more concrete evidence of direct physical interaction between *Cel*-EAT-2 and *Cel*-EAT-18. EAT-18 modifies the pharmacological properties of the EAT-2 nAChR and is necessary for *in vivo* cholinergic MC neurotransmission which regulates pharyngeal

pumping. Therefore EAT-18 meets the criteria of an auxiliary protein. However, unlike other auxiliary proteins, successful expression of a functioning channel requires the presence of EAT-18. Various auxiliary subunits have been discovered for the ionotropic glutamate-gated (kainate and AMPA) and $GABA_B$ receptors that modulate their properties or functional expression levels (Yan and Tomita 2012; Ben-Yaacov et al. 2017; Schwenk et al. 2010; Tomita and Castillo 2012). The interaction of these auxiliary subunits with the receptor complex holds a physiological relevance. MOLO-1 (modulator of levamisole receptor-1) is the first example of an auxiliary subunit for levamisole sensitive acetylcholine receptors in *C. elegans* (Boulin et al. 2012). Like MOLO-1, null mutants of EAT-18 result in significant physiological defects and many of the nematode species express highly conserved orthologues of EAT-18 (Supplementary Information S3.8) suggesting evolutionary conservation of function. In contrast to EAT-18, MOLO-1 is not required for the functional expression of the somatic levamisole nAChR but only regulated the trafficking, localization or gating kinetics; instead EAT-18 is essential for EAT-2 to form a functional receptor *in vitro*. This implies that EAT-18 not only meets the criteria for an auxiliary protein but may belong to a novel class of proteins not previously described, adding to the types of auxiliary subunits identified for the cys-loop cation channels.

Identification of a suitable target and its validation is one of the most important steps in developing a new drug. An ideal anthelmintic target should meet certain criteria in order to be considered relevant for pharmacological intervention; important physiological function, conservation across parasite species and pharmacological divergence from host receptors. Parasite nicotinic acetylcholine receptors are considered

as popular targets because they contribute to vital physiological functions. Additionally, their diversity, conserved structure among various species of nematodes and distinct pharmacology from mammalian orthologues makes them "druggable". The pharynx is a muscular organ required for feeding in nematodes. While the nematode pharynx has been exploited as a target tissue for the avermectins ($GluCl^-$ channels) (Cully et al. 1994a; Martin 1996; Brownlee, Holden-Dye, and Walker 1997; Pemberton et al. 2001) less is known about the nAChRs in this tissue. In *C. elegans* two genes, *eat-2* and *eat-18,* were required for MC neurotransmission. *Cel*-EAT-2 and *Cel*-EAT-18 are both localized in the pharyngeal muscles and mutations in these genes caused defects in feeding behavior in the worms (Raizen, Lee, and Avery 1995; McKay et al. 2004). We hypothesize that activation of the pharyngeal nAChR formed by the EAT-2 subunit and EAT-18 auxiliary protein will lead to an effect similar to levamisole in somatic muscle and cause pharyngeal paralysis in nematodes. We were able to successfully co-express EAT-2 and EAT-18 from *C. elegans*, a model nematode, and *A.* suum, a parasitic species, in *Xenopus* oocytes and characterize the pharmacology of this conserved receptor.

3.5.1 The pharyngeal nAChR composed of EAT-2 and EAT-18 as a novel drug target

The pharyngeal cys-loop ligand-gated ion channel formed by EAT-2 meets the criteria for a suitable anthelmintic drug target (Wever, Farrington, and Dent 2015): 1) it performs a neuromuscular function essential for parasite biology; 2) this receptor is druggable, it has distinct pharmacology from somatic muscle receptors and is insensitive to many of the currently used cholinergic anthelmintic including morantel, tribendimidine and pyrantel; 3) EAT-2 and EAT-18 are present in multiple relevant parasitic nematode

species (Supplementary Information S3.7 and S3.8) and the protein sequences are highly conserved; 4) *Cel*-EAT-2 is only 36% identical to human α-7 nAChR subunit and there are no mammalian homologues for EAT-18 providing potential for selectivity; it is also pharmacologically distinct from vertebrate nAChRs. In order to identify novel drug targets for anthelmintic agents, it is important to understand the properties and function of the target proteins. We have successfully elucidated the components and pharmacological profile of the pharyngeal nAChR by employing various molecular, biochemical and electrophysiology techniques.

3.6 Acknowledgements

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3.7 Contributions

Conceived and designed study: A.P.R., S.C., S.K.B., G.R.M & RJM. Conducted experiments: S.C., S.P., S.V., C.J.M., B.J.R. Analyzed data: S.C., A.P.R. & S.P. Wrote manuscript: A.P.R., S.C., G.R.M., A.J.W. & R.J.M.

3.8 Competing interests

The authors declare no competing interests.

3.9 References

Abongwa, M., S. K. Buxton, E. Courtot, C. L. Charvet, C. Neveu, C. J. McCoy, S. Verma, A. P. Robertson, and R. J. Martin. 2016. 'Pharmacological profile of Ascaris suum ACR-16, a new homomeric nicotinic acetylcholine receptor widely distributed in Ascaris tissues', *Br J Pharmacol*, 173: 2463-77.

Abongwa, M., R. J. Martin, and A. P. Robertson. 2017. 'A Brief Review on the Mode of Action of Antinematodal Drugs', *Acta Vet (Beogr)*, 67: 137-52.

Albertson, D. G., and J. N. Thomson. 1976. 'The pharynx of Caenorhabditis elegans', *Philos Trans R Soc Lond B Biol Sci*, 275: 299-325.

Avery, L., and H. R. Horvitz. 1990. 'Effects of starvation and neuroactive drugs on feeding in Caenorhabditis elegans', *J Exp Zool*, 253: 263-70.

Ballivet, M., C. Alliod, S. Bertrand, and D. Bertrand. 1996. 'Nicotinic acetylcholine receptors in the nematode Caenorhabditis elegans', *J Mol Biol*, 258: 261-9.

Ben-Yaacov, Anat, Moshe Gillor, Tomer Haham, Alon Parsai, Mohammad Qneibi, and Yael Stern-Bach. 2017. 'Molecular Mechanism of AMPA Receptor Modulation by TARP/Stargazin', *Neuron*, 93: 1126-37.e4.

Bianchi, L., and M. Driscoll. 2006. "Heterologous expression of C. elegans ion channels in Xenopus oocytes. ." In *WormBook*, 1-16. The *C. elegans* Research Community, WormBook.

Boulin, T., M. Gielen, J. E. Richmond, D. C. Williams, P. Paoletti, and J. L. Bessereau. 2008. 'Eight genes are required for functional reconstitution of the Caenorhabditis elegans levamisole-sensitive acetylcholine receptor', *Proc Natl Acad Sci U S A*, 105: 18590-5.

Boulin, T., G. Rapti, L. Briseno-Roa, C. Stigloher, J. E. Richmond, P. Paoletti, and J. L. Bessereau. 2012. 'Positive modulation of a Cys-loop acetylcholine receptor by an auxiliary transmembrane subunit', *Nat Neurosci*, 15: 1374-81.

Brownlee, D. J., L. Holden-Dye, and R. J. Walker. 1997. 'Actions of the anthelmintic ivermectin on the pharyngeal muscle of the parasitic nematode, Ascaris suum', *Parasitology*, 115 (Pt 5): 553-61.

Buxton, Samuel K., Claude L. Charvet, Cedric Neveu, Jacques Cabaret, Jacques Cortet, Nicolas Peineau, Melanie Abongwa, Elise Courtot, Alan P. Robertson, and Richard J. Martin. 2014. 'Investigation of Acetylcholine Receptor Diversity in a Nematode Parasite Leads to Characterization of Tribendimidine- and Derquantel-Sensitive nAChRs', *PLOS Pathogens*, 10: e1003870.

Chen, D., and J. W. Patrick. 1997. 'The alpha-bungarotoxin-binding nicotinic acetylcholine receptor from rat brain contains only the alpha7 subunit', *J Biol Chem*, 272: 24024-9.

Choudhary, Shivani, Djordje S. Marjianović, Colin R. Wong, Xiaoyu Zhang, Melanie Abongwa, Joel R. Coats, Saša M. Trailović, Richard J. Martin, and Alan P. Robertson. 2019. 'Menthol acts as a positive allosteric modulator on nematode levamisole sensitive nicotinic acetylcholine receptors', *International Journal for Parasitology: Drugs and Drug Resistance*, 9: 44-53.

Corringer, P. J., N. Le Novere, and J. P. Changeux. 2000. 'Nicotinic receptors at the amino acid level', *Annu Rev Pharmacol Toxicol*, 40: 431-58.

Cully, D. F., D. K. Vassilatis, K. K. Liu, P. S. Paress, L. H. Van der Ploeg, J. M. Schaeffer, and J. P. Arena. 1994. 'Cloning of an avermectin-sensitive glutamate-gated chloride channel from Caenorhabditis elegans', *Nature*, 371: 707-11.

Decker, Michael W., Jorge D. Brioni, Anthony W. Bannon, and Stephen P. Arneric. 1995. 'Diversity of neuronal nicotinic acetylcholine receptors: Lessons from behavior and implications for cns therapeutics', *Life Sciences*, 56: 545-70.

Greenberg, R. M. 2014. 'Ion channels and drug transporters as targets for anthelmintics', *Curr Clin Microbiol Rep*, 1: 51-60.

Haugstetter, J., T. Blicher, and L. Ellgaard. 2005. 'Identification and characterization of a novel thioredoxin-related transmembrane protein of the endoplasmic reticulum', *J Biol Chem*, 280: 8371-80.

Holden-Dye, L., M. Joyner, V. O'Connor, and R. J. Walker. 2013. 'Nicotinic acetylcholine receptors: a comparison of the nAChRs of Caenorhabditis elegans and parasitic nematodes', *Parasitol Int*, 62: 606-15.

J Brownlee, D., Lindy Holden-Dye, Robert Walker, and Ian Fairweather. 1995. *The pharynx of the nematode Ascaris suum: structure and function*.

Jones, A. K., and D. B. Sattelle. 2004. 'Functional genomics of the nicotinic acetylcholine receptor gene family of the nematode, Caenorhabditis elegans.', *Bioessays*, 26: 39-49.

Kaminsky, R., P. Ducray, M. Jung, R. Clover, L. Rufener, J. Bouvier, S. S. Weber, A. Wenger, S. Wieland-Berghausen, T. Goebel, N. Gauvry, F. Pautrat, T. Skripsky, O. Froelich, C. Komoin-Oka, B. Westlund, A. Sluder, and P. Maser. 2008. 'A new class of anthelmintics effective against drug-resistant nematodes', *Nature*, 452: 176-80.

Kao, P. N., A. J. Dwork, R. R. Kaldany, M. L. Silver, J. Wideman, S. Stein, and A. Karlin. 1984. 'Identification of the alpha subunit half-cystine specifically labeled by an

affinity reagent for the acetylcholine receptor binding site', *J Biol Chem*, 259: 11662-5.

Kao, P. N., and A. Karlin. 1986. 'Acetylcholine receptor binding site contains a disulfide cross-link between adjacent half-cystinyl residues', *J Biol Chem*, 261: 8085-8.

Karlin, A. 2002. 'Emerging structure of the nicotinic acetylcholine receptors', *Nat Rev Neurosci*, 3: 102-14.

Lin-Moshier, Y., and J. S. Marchant. 2013. 'A rapid Western blotting protocol for the Xenopus
oocyte.', *Cold Spring Harb Protoc.*, 3.

Little, P. R., A. Hodge, S. J. Maeder, N. C. Wirtherle, D. R. Nicholas, G. G. Cox, and G. A. Conder. 2011. 'Efficacy of a combined oral formulation of derquantel-abamectin against the adult and larval stages of nematodes in sheep, including anthelmintic-resistant strains', *Vet Parasitol*, 181: 180-93.

Martin, R. J. 1985. 'gamma-Aminobutyric acid- and piperazine-activated single-channel currents from Ascaris suum body muscle', *Br J Pharmacol*, 84: 445-61.

———. 1996. 'An electrophysiological preparation of Ascaris suum pharyngeal muscle reveals a glutamate-gated chloride channel sensitive to the avermectin analogue, milbemycin D', *Parasitology*, 112: 247-52.

McKay, J. P., D. M. Raizen, A. Gottschalk, W. R. Schafer, and L. Avery. 2004. 'eat-2 and eat-18 are required for nicotinic neurotransmission in the Caenorhabditis elegans pharynx', *Genetics*, 166: 161-9.

Millar, N. S. 2008. 'RIC-3: a nicotinic acetylcholine receptor chaperone', *Br J Pharmacol*, 153 Suppl 1: S177-83.

Pemberton, D. J., C. J. Franks, R. J. Walker, and L. Holden-Dye. 2001. 'Characterization of glutamate-gated chloride channels in the pharynx of wild-type and mutant Caenorhabditis elegans delineates the role of the subunit GluCl-alpha2 in the function of the native receptor', *Mol Pharmacol*, 59: 1037-43.

Raizen, D. M., R. Y. Lee, and L. Avery. 1995a. 'Interacting genes required for pharyngeal excitation by motor neuron MC in Caenorhabditis elegans', *Genetics*, 141: 1365-82.

Raizen, D. M., R. Y. N. Lee, and L. Avery. 1995b. 'Interacting Genes Required for Pharyngeal Excitation by Motor Neuron Mc in Caenorhabditis Elegans', *Genetics*, 141: 1365-82.

Raymond, V., N. P. Mongan, and D. B. Sattelle. 2000. 'Anthelmintic actions on homomer-forming nicotinic acetylcholine receptor subunits: chicken alpha7 and ACR-16 from the nematode Caenorhabditis elegans', *Neuroscience*, 101: 785-91.

Robertson, A. P., H. E. Bjorn, and R. J. Martin. 2000. 'Pyrantel resistance alters nematode nicotinic acetylcholine receptor single-channel properties', *Eur J Pharmacol*, 394: 1-8.

Robertson, A. P., S. K. Buxton, and R. J. Martin. 2013. 'Whole-cell patch-clamp recording of nicotinic acetylcholine receptors in adult Brugia malayi muscle', *Parasitology International*, 62: 616-18.

Robertson, A. P., C. L. Clark, T. A. Burns, D. P. Thompson, T. G. Geary, S. M. Trailovic, and R. J. Martin. 2002. 'Paraherquamide and 2-deoxy-paraherquamide distinguish cholinergic receptor subtypes in Ascaris muscle', *J Pharmacol Exp Ther*, 302: 853-60.

Schwenk, Jochen, Michaela Metz, Gerd Zolles, Rostislav Turecek, Thorsten Fritzius, Wolfgang Bildl, Etsuko Tarusawa, Akos Kulik, Andreas Unger, Klara Ivankova, Riad Seddik, Jim Y. Tiao, Mathieu Rajalu, Johana Trojanova, Volker Rohde, Martin Gassmann, Uwe Schulte, Bernd Fakler, and Bernhard Bettler. 2010. 'Native GABAB receptors are heteromultimers with a family of auxiliary subunits', *Nature*, 465: 231.

Seymour, Malcolm K., K. A. Wright, and C. C. Doncaster. 1983. 'The action of the anterior feeding apparatus of Caenorhabditis elegans (Nematoda: Rhabditida)', *Journal of Zoology*, 201: 527-39.

Tomita, Susumu, and Pablo E. Castillo. 2012. 'Neto1 and Neto2: auxiliary subunits that determine key properties of native kainate receptors', *The Journal of Physiology*, 590: 2217-23.

Tompkins, J. B., L. E. Stitt, and B. F. Ardelli. 2010. 'Brugia malayi: in vitro effects of ivermectin and moxidectin on adults and microfilariae', *Exp Parasitol*, 124: 394-402.

Wever, Claudia M., Danielle Farrington, and Joseph A. Dent. 2015. 'The Validation of Nematode-Specific Acetylcholine-Gated Chloride Channels as Potential Anthelmintic Drug Targets', *PLOS ONE*, 10: e0138804.

White, J. G., E. Southgate, J. N. Thomson, and S. Brenner. 1986. 'The structure of the nervous system of the nematode Caenorhabditis elegans', *Philos Trans R Soc Lond B Biol Sci*, 314: 1-340.

WHO. 2018. 'Soil-transmitted helminth infections'. http://www.who.int/news-room/fact-sheets/detail/soil-transmitted-helminth-infections.

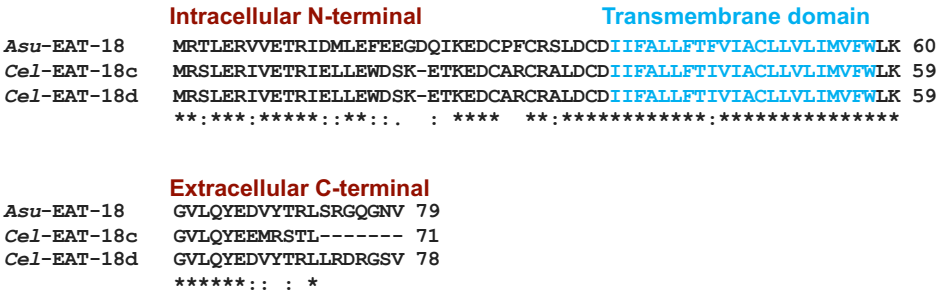
Wolstenholme, A. J. 2011. 'Ion channels and receptor as targets for the control of parasitic nematodes', *Int J Parasitol Drugs Drug Resist*, 1: 2-13.

Wolstenholme, A. J., and A. T. Rogers. 2005. 'Glutamate-gated chloride channels and the mode of action of the avermectin/milbemycin anthelmintics', *Parasitology*, 131 Suppl: S85-95.

Xiao, Shu-Hua, Wu Hui-Ming, Marcel Tanner, Jürg Utzinger, and Wang Chong. 2005. 'Tribendimidine: a promising, safe and broad-spectrum anthelmintic agent from China', *Acta Tropica*, 94: 1-14.

Yan, D., and S. Tomita. 2012. 'Defined criteria for auxiliary subunits of glutamate receptors', *J Physiol*, 590: 21-31.

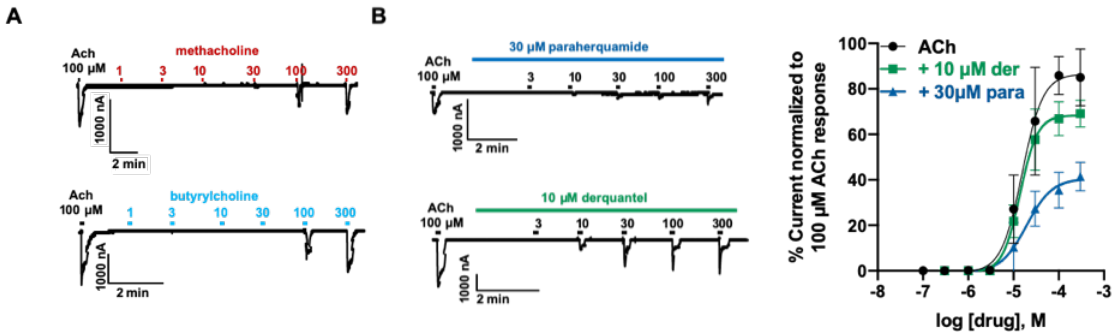
3.10 Supporting information



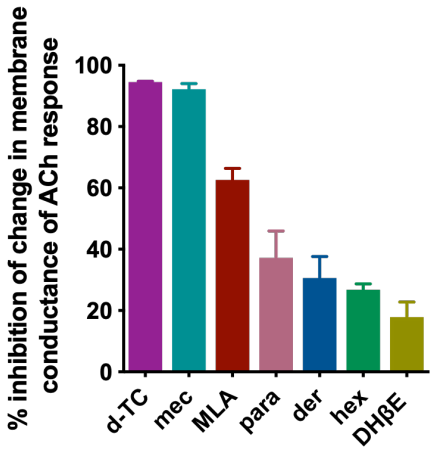
Supporting Figure S3.0 Amino acid sequence alignment of *Asu*-EAT-18, *Cel*-EAT-18c, and *Cel*-EAT-18d. Predicted transmembrane domain is highlighted in blue.

Supporting Figure S3.1 Amino acid sequence alignment of *Cel*-EAT-2, *Asu*-EAT-2 and human-α7 nAChR subunits. The signal peptide (olive green), ACh-binding loops A–C (purple), cys-loop (orange) and transmembrane regions TM1–TM4 (light blue) are indicated. The vicinal cysteines (grey box) are absent in the C-binding loop of the EAT-2 protein. The conserved ligand binding residues of human-α7 subunits are highlighted in blue color in loops A-C and in maroon color in loops D-F. The residues

not conserved in EAT-2 proteins are in grey boxes in the loops. The negatively charged acid residues flanking the transmembrane-2 region are highlighted in orange.

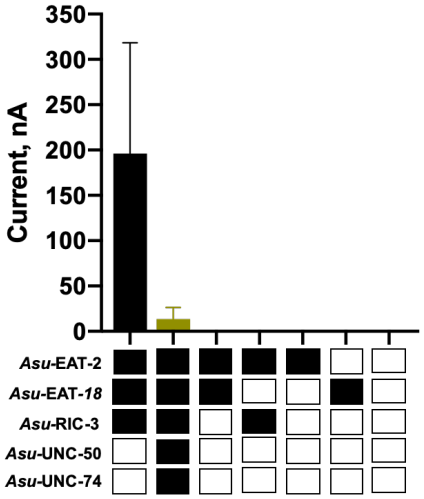


Supporting Figure S3.2 Pharmacology of the *Cel*-EAT-2 nicotinic acetylcholine receptor. A. Representative traces for methacholine and butyrylcholine concentration-response relationships for *Cel*-EAT-2 receptor. B. Representative traces & acetylcholine concentration-response curves for *Cel*-EAT-2 receptor in the presence of 10 µM derquantel (der, n=6) and 30 µM paraherquamide (para, n=6). The pEC_{50} and I_{max} values (expressed as mean±SEM) were: 4.9±0.0 and 68.4±2.1% in the presence of derquantel; 4.7±0.1 and 40.2±2.7% in the presence of 30 µM paraherquamide. Both the antagonists did not produce a shift in pEC_{50} but reduced the efficacy of the acetylcholine on the *Cel*-EAT-2 receptor significantly ($^{****}P < 0.0001$, Extra sum of squares F-test).

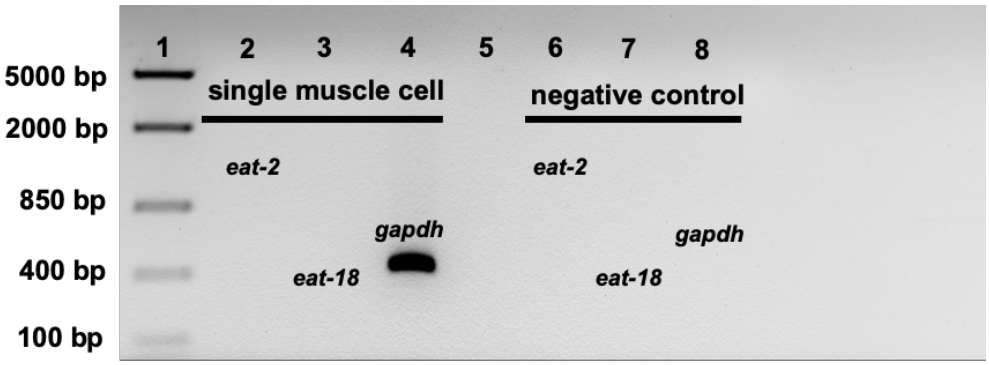


Supporting Figure S3.3 Bar graph showing the rank order potency of selected vertebrate nAChR antagonists (30µM) producing % inhibition of 100µM ACh membrane conductance (δG; expressed as mean ± SEM, %) in the *A. suum* pharynx: d-Tubocurarine (d-TC; 94.6±0.2) > mecamylamine (mec; 92.2±1.9) > methyllycaconitine (MLA; 62.6±3.7) > paraharquamide (para; 37.2±8.7) > derquantel

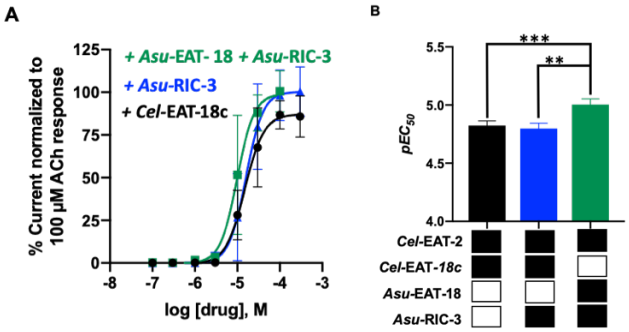
(der; 30.6±7.0) > hexamethonium (hexa; 26.8±1.9) > dihydro-β-erythroidine (DHβE; 17.9±5.0).



Supporting Figure S3.4 Bar chart showing current sizes (mean ± S.E.M) produced in response to 100 µM acetylcholine on *Asu*-EAT-2 nAChR. Black bar: *Asu*-EAT-2 + *Asu*-EAT-18 + *Asu*-RIC-3 (n=11). Olive green bar: *Asu*-EAT-2 + *Asu*-EAT-18 + *Asu*-RIC-3 + *Asu*-UNC-50 + *Asu*-UNC-74 (n=6). *Asu*-EAT-2 and *Asu*-EAT-18 did not form a functioning receptor on their own. Un-injected oocytes were used as negative control.



Supporting Figure S3.5 Single-cell RT-PCR of *Asu-eat-2* (lanes 2, 6), *Asu-eat-18* (lanes 3, 7) and gapdh control (lanes 4,8) in somatic muscle cells (n=10). Lane 1, FastRuler High Range DNA ladder; negative control- no-template controls for *Asu-eat-2* (lane-6), *Asu-eat-18* (lanes 7) and gapdh (lane-8).

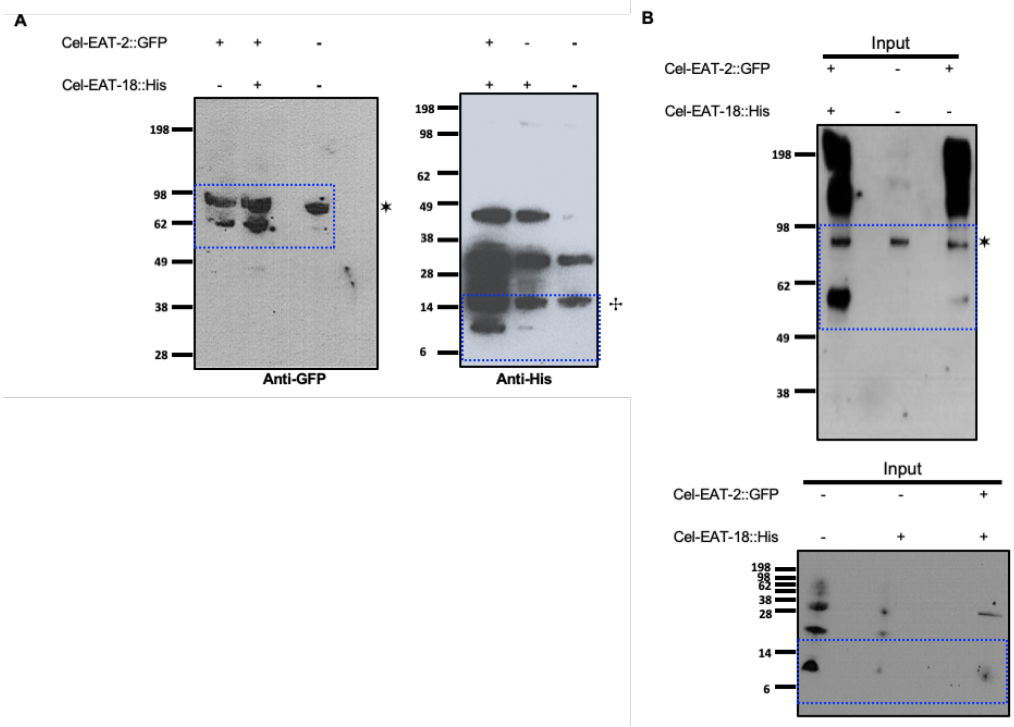


Supporting Figure S3.6 A. Concentration response curves for acetylcholine application on *Cel*-EAT-2 + *Cel*-EAT-18c mix (black curve), *Cel*-EAT-2 + *Cel*-EAT-18c + *Asu*-RIC-3 (blue curve) and *Cel*-EAT-2 + *Asu*-EAT-18 + *Asu*-RIC-3 mix (green curve). B. Bar graphs showing significant effect of using different EAT-18 proteins with *Cel-eat-2* on pEC_{50}. $^{**}P < 0.01$, $^{***}P < 0.001$; significantly different as indicated; based on Extra sum of squares F-test.

Supporting Figure S3.6 Protein sequence alignment of EAT-2 subunit from multiple parasitic nematode species. The signal peptide (olive green), ACh-binding loops A–C (pink), loops D-F (green), cys-loop (grey) and transmembrane regions TM1–TM4 (blue) are indicated. The conserved ligand binding residues are highlighted in blue color in loops A-C and in maroon color in loops D-F.

```
                    Intracellular N-terminal                                  Transmembrane domain
S.stercoralis       MVSEYQARTLENVVDARIEMLQFEKEEDEEVEEDCSFCRSLDCDILFALSFTLIIIGLLI
E.vermicularis      ------MRTLERVVETRIDMLEFEKA--DEVKEDCSFCRSLDCDIIFALLFTFVIGCLLV
T.canis             ------MRTLERVVETRIDMLQFEKG--DQIKEDCPFCRSLDCDIIFALLFTFVIACLLV
A.suum              ------MRTLERVVETRIDMLEFEEG--DQIKEDCPFCRSLDCDIIFALLFTFVIACLLV
C.elegans           ------MRSLERIVETRIELLEWDSK---ETKEDCARCRALDCDIIFALLFTIVIACLLV
A.ceylanicum        ------MRTLERVVETRIDLLEWESP---APKEDCAACRALDCDIIFALLFTMVIACLLV
                           *:**.:*::**::*:::.      :***  **:*****:*** **::*  **:
                    Extracellular C-terminal
S.stercoralis       LIVIFWLKGVMQYQDVYTRLSPEKDNA-
E.vermicularis      LIMAFWLKGVVQYKDVYTRLSHGRTNAR
T.canis             LIMVFWLKGVLQYEDVYTRLSRGQGNA-
A.suum              LIMVFWLKGVLQYEDVYTRLSRGQGNV-
C.elegans           LIMVFWLKGVLQYEDVYTRLLRDRGSV-
A.ceylanicum        LIMVFWLKGVLQYEDVYTRLSRDRGNA-
                    **: ******:**:******   : ..
```

Supporting Figure S3.7 Amino acid sequence alignment of EAT-18 from multiple parasitic nematode species. Transmembrane domain highlighted in blue.



Supporting Figure S3.8: A. Uncropped western blots corresponding to Figure 3.4E. B. Uncropped western blots corresponding to Figure 3.4f. Dashed blue regions represent the cropped regions used in the main figures.

Ascaris suum				
nAChRs		**Nicotinic agonists**	**Nicotinic antagonists**	**Cholinergic anthelmintics**
Pharyngeal		ACh > nic > cyt > epi > DMPP > chol	d-TC > mec > MLA > para > der > hexa > DHβE	ACh > bep > the > lev ≅ mor ≅ pyr ≅ oxa ≅ met ≅ tri
Somatic muscle		DMPP > ACh > nic [1]	mec > d-TC > hexa > DHβE [1]	pyr > oxa > bep > the > lev > ACh > nic > met [2,3]
Vertebrates				
Skeletal muscle	**Adult**	cyt > DMPP > nic [4]		
CNS/ Neuronal	**α4β2**	epi > cyt > DMPP > nic > ACh [5]	DHβE > MLA [7]	
	α3β4	epi > cyt > DMPP > nic > ACh [5]	mec > d-TC > DHβE > hexa [5]	
	α7	cyt > nic > ACh > cho [6]	MLA > mec > DHβE [6]	

Supplementary Table 1: Rank order potencies of nAChR agonists, antagonists and cholinergic anthelmintics in *A. suum* pharyngeal nAChRs observed from our study, *A. suum* somatic muscle nAChRs and nAChRs of the vertebrate hosts. ACh (acetylcholine), nic (nicotine), cyt (cytisine), epi (epibaditine), DMPP (dimethylphenylpiperazine), chol (choline), pyr (pyrantel), oxa (oxantel), bep (bephenium), the (thenium), lev (levamisole), met (methyridine), d-TC (d-tubocurarine), mec (mecamylamine), MLA (methyllycaconitine), para (paraherquamide), der (derquantel), hexa (hexamethonium) and DHβE (Dihydro-β-erythroidine).

[1] Colquhoun, L., Holden-Dye, L. & Walker, R.J. *J Exp Biol* **158**, 509-530 (1991).
[2] Martin, R.J., Clark, C.L., Trailovic, S.M. & Robertson, A.P. *Int J Parasitol* **34**, 1083-1090 (2004).
[3] Trailovic, S.M., Verma, S., Clark, C.L., Robertson, A.P. & Martin, R.J. *Int J Parasitol* **38**, 945-957 (2008).
[4] Yost, C.S. & Winegar, B.D. *Cell Mol Neurobiol* **17**, 35-50 (1997) .
[5] Wonnacot, S. & Barik, J. *Tocris Bioscience scientific review series* **28,**1-20 (2007).
[6] Virginio, C., Giacometti, A., Aldegheri, L., Rimland, J.M. & Terstappen, G.C. Eur J Pharmacol **445**, 153-161 (2002).
[7]Buisson, B., Gopalakrishnan, M., Arneric, S.P., Sullivan, J.P. & Bertrand, D. *J Neurosci*. **16**(24):7880-91(1996).

Supplementary Data 1: Acetylcholine is a known agonist at nicotinic and muscarinic receptors in vertebrates. The rank order potency of muscarinic agonists (all used at 100μM) observed for an *A. suum* muscarinic receptor expressed in yeast was ACh (100%) > carbachol (80%) ≅ arecoline (76%) > oxotremorine (61%) ≅ bethanechol (58%) > pilocarpine (14%)[1]. Here we tested the hypothesis that the observed change in membrane conductance response (δG) to ACh on the pharynx is produced by activation of nAChRs and mAChRs (GARs). Our pharyngeal preparations in this group had a resting membrane potential (RMP) of -17.6±1.3 mV and a resting conductance of 147.8±13.1 μS (n=8, mean±SE). We tested responses to the muscarinic agonists ACh, 5-methylfurmethiodide (MFI), oxotremorine, arecoline, pilocarpine (all 100μM, 10s application). In applications containing arecoline and pilocarpine, we used mecamylamine, a nAChR antagonist (30μM) to inhibit nAChRs and to allow only mAChR activation. A control application of ACh (100 μM, 10s) in the presence of mecamylamine was used for comparison of response to nAChR activation. We normalized the conductance change (δG) produced by the control ACh application (100 μM, 10s) to 100% in order compare the relative responses to other muscarinic agonists. The normalized responses to muscarinic agonists (mean ± SE, %, n=4) were MFI (4.0±0.6), oxotremorine (1.8±0.8), arecoline (2.6±2.0), pilocarpine (0.7±0.7). Mecamylamine inhibited 92% of the ACh δG response suggesting nicotinic receptor activation rather than the muscarinic receptor activation was responsible for the observed changes. Our results demonstrate the contribution of mAChRs to the acetylcholine induced conductance changes is negligible. Therefore, it was unnecessary to further use muscarinic receptor antagonists in our experiments to characterize the nAChRs. Other work has documented the phenomenon of concentration dependent reversible channel block produced by atropine (IC50 = 4-10μM) on α3β4, α3β2, α4β4 and α4β2 vertebrate nAChRs[2].

[1] Kimber M.J. et al. *Int J Parasitol* 39: 1215-1222 (2009).
[2] Parker J.C., Sarkar D., Quick M.W. & Lester R.A. *Br J Pharmacol* 138: 801-810 (2003).

CHAPTER 4. PHARMACOLOGICAL CHARACTERIZATION OF A HOMOMERIC NICOTINIC ACETYLCHOLINE RECEPTOR FORMED BY *ANCYLOSTOMA CANINUM* ACR-16

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4.1 Abstract

Parasitic nematode infections are treated using anthelmintic drugs, some of which target nicotinic acetylcholine receptors (nAChRs) located in different parasite tissues. The limited arsenal of anthelmintic agents and the prevalence of drug resistance implies that future defense against parasitic infections will depend on the discovery of novel targets and therapeutics. Previous studies have suggested that *Ascaris suum* ACR-16 nAChRs are a suitable target for the development of antinematodal drugs. In this study we characterized the pharmacology of the *Ancylostoma caninum* ACR-16 receptor using two-electrode voltage-clamp electrophysiology. This technique allowed us to study the effects of cholinergic agonists and antagonists on the nematode nAChRs expressed in *Xenopus laevis* oocytes. *Aca*-ACR-16 was not sensitive to many of the existing cholinomimetic anthelmintics (levamisole, oxantel, pyrantel and tribendimidine). 3-Bromocytisine was the most potent agonist (>130% of the control acetylcholine current) on the *Aca*-ACR-16 nAChR but, unlike *Asu*-ACR-16, oxantel did not activate the

receptor. The mean time constants of desensitization for agonists on *Aca*-ACR-16 were longer than the rates observed in *Asu*-ACR-16. In contrast to *Asu*-ACR-16, the *A. caninum* receptor was completely inhibited by DhβE and moderately inhibited by α-BTX. In conclusion, we have successfully reconstituted a fully functional homomeric nAChR, ACR-16, from *A. caninum*, a model for human hookworm infections. The pharmacology of the receptor is distinct from levamisole sensitive nematode receptors. The ACR-16 homologue also displayed some pharmacological differences from *Asu*-ACR-16. Hence, *A. caninum* ACR-16 may be a valid target site for the development of anthelmintics against hookworm infections.

Keywords

nAChR, hookworms, *Aca*-ACR-16, anthelmintic, *Xenopus* oocyte

4.2 Introduction

Infections caused by hookworms (mainly *Necator americanus* and *Ancylostoma duodenale*) are one of the leading neglected tropical disease, affecting approximately 500 million people worldwide; especially in the developing regions of Asia, Africa, Latin America and the Caribbean (Pullan et al. 2014; Loukas et al. 2016; Hotez 2008). These infections account for >4 million disability adjusted life years (DALYs) lost annually and an estimated global economic loss of over US $100 billion (Loukas et al. 2016; Bartsch et al. 2016). These blood feeding nematodes do not directly account for substantial mortality; instead the major clinical manifestations of hookworm infection are the consequences of chronic intestinal blood loss. Severe infection can result in iron deficiency, anemia, weight loss, abdominal pain, protein loss, and diarrhea (Bethony et

al. 2006; Hotez and Pritchard 1995). Hookworm infections pose a major health threat to adolescent girls, women of reproductive age and children (Menzies et al. 2014; Brooker, Hotez, and Bundy 2008). Heavy worm burdens can result in retarded physical and cognitive development in children and poor outcomes for pregnant women and their newborns (Guyatt et al. 2000; de Silva et al. 2003a; Hotez et al. 2014).

Current hookworm control strategies are limited to deworming of infected people using anthelmintic drugs combined with ancillary strategies such as improvement of water quality, sanitation and hygiene (WASH) in endemic regions (WHO 2015; Campbell et al. 2018; Albonico et al. 2003). At this time, there is no effective vaccine for human use in medical circulation (Hewitson and Maizels 2014; Diemert, Bethony, and Hotez 2008), and only a limited number of drug options. Unfortunately, there have been failures of mass drug administration in endemic regions due to diminished efficacy and potential resistance to anthelmintic agents (Albonico et al. 2003; Krücken et al. 2017; Flohr et al. 2007; De Clercq et al. 1997; Reynoldson et al. 1997). The rapid reinfection rate of the worm combined with the ability of adult hookworms to survive up to 7 years in the human gut producing thousands of ova per day further complicates the issue (Albonico et al. 1995; Bennett and Guyatt 2000; Knopp et al. 2012). Due to all of these contributing factors, novel drug targets and drugs are required for efficient control of these parasitic infections.

Research has focused on several different parasite ion channels because they are major target sites of many classes of antinematodal agents (Wolstenholme 2011; Abongwa, Martin, and Robertson 2017). Ion channels are essential for fundamental physiological functioning in gastrointestinal worms. Nicotinic acetylcholine receptors

(nAChRs) which belong to the cys-loop ligand gated ion channel family serve as synaptic transmission proteins and mediate fast transduction of signals by opening an intrinsic ion channel (Jones et al. 2007; Thompson, Lester, and Lummis 2010a). They are pentameric channels which can be homomeric or heteromeric around a central pore. Nicotinic anthelmintics such as pyrantel and levamisole selectively paralyze nematodes by activating cholinergic ion-channels (nAChRs) in their body wall muscle (Abongwa, Martin, and Robertson 2017; Aceves, Erlij, and Martínez-Marañón 1970; Harrow and Gration 1985; Aubry et al. 1970; Martin et al. 2005). The significance of nematode nAChRs as drug targets has been emphasized by the recent development of novel amino-acetonitrile compounds (Kaminsky et al. 2008).

Ancylostoma caninum is the most widespread and pathogenic hookworm of dogs (Nemzek et al. 2015). Infestation typically results in anemia with bloody diarrhea, hemorrhagic enteritis, vomiting, anorexia, dehydration and poor weight gain, sometimes leading to death (Epe 2009; Dias et al. 2013). Zoonotic infection with *A. caninum* in humans has been associated with eosinophilic enteritis, localized myositis and cutaneous larva migrans (Prociv and Croese 1996; Bowman et al. 2010; Landmann and Prociv 2003; Traversa 2012). *Ancylostoma caninum* is the most accessible of all hookworms for research and is closely related to human hookworm species, *A. duodenale* and *N. americanus.* Therefore, they are used as a model for human hookworm (Nemzek et al. 2015; Prociv and Croese 1996; Blaxter 2000).

In this study, we have cloned and expressed a homologue of ACR-16 from *A. caninum*, a clade V nematode parasite. The receptors were expressed in *Xenopus laevis* oocytes and we used two-electrode voltage-clamp electrophysiology to characterize their

pharmacology. ACR-16 has been suggested as a druggable target in the parasitic clade III roundworm, *Ascaris suum* by Abongwa, Buxton, et al. (2016). The focus of this study was to generate a comparative pharmacological analysis of the homomeric channel and establish ACR-16 as a potential target in the hookworm parasites.

4.3 Materials and Methods

4.3.1 Ethical concerns

No vertebrate animals were used directly in this study. Defolliculated *Xenopus laevis oocytes* were obtained from Ecocyte Bioscience (Austin, TX, USA).

4.3.2 Parasites

Ancylostoma caninum were obtained opportunistically from a naturally infected dog. Feces containing eggs was mixed with vermiculite and stored in a humidified container for 8 days. The mixture was overlaid with cheesecloth and was placed under a desktop lamp at room temperature for 8 hours. L3 larvae were then concentrated and collected by the Baermann method (Hawdon 1991).

4.3.3 Sequence analysis

Database searches for *A. caninum* ACR-16 were performed by BLAST search (WormBase Parasite), using the BLASTP algorithms (Altschul et al. 1997). Signal peptide predictions were done using the SignalP 4.1 server (Petersen et al. 2011), and membrane-spanning regions were identified using TMpred (Hofmann and Stoffel 1993). Alignment of the full-length amino acid sequences with *Ascaris suum* ACR-16 was carried out using Clustal Omega program (Sievers et al. 2011).

4.3.4 Cloning of *Aca*-ACR-16

TRIzol Reagent™ (Invitrogen™, Carlsbad, CA, USA) was used to extract total RNA from homogenized *A. caninum* larvae. cDNA was synthesized by using SuperScript VILO Master Mix (Invitrogen™, Carlsbad, CA, USA) according to the manufacturer's instructions and served as a template for *Aca*-ACR-16 amplification (WormBase Parasite Gene ID: ANCCAN_01899). We used the Gibson assembly protocol to assemble the amplified fragments into the full length *Aca*-ACR-16 sequence (Gibson et al. 2009). Full length product was subcloned into pTB207 expression vector by adding *XhoI* and *ApaI* restriction enzyme sites respectively to the forward primer (5' end: TGGCGGCCGctcgagATGCGTTCGTTGGTCGTCTG) and reverse primers (3' end: ATCAAGCTCgggcccTTAGGCGACGAGATATGGAGC) using In-Fusion cloning (Takara Bio USA, Inc.). Z-competent *E. coli* JM109 cells (Zymo Research, Irvine, CA) were used for transformation of the ligated product. The final cloned constructs were sequenced with pTB207 vector primers (forward, T7) and (reverse, SP6). Only positive clones were used for cRNA synthesis using *in vitro* transcription with the mMessage mMachine T7 transcription kit (Invitrogen, CA, USA) and the cRNA was aliquoted and stored at -80°C.

4.3.5 Oocyte microinjection and electrophysiology

Xenopus laevis oocyte injections and two-electrode voltage-clamp electrophysiology recordings were performed as previously described in Choudhary et al. (2019). The oocytes were injected with 25-50 ng of *Aca-ACR*-16 cRNA either alone or in combination with 15-25 ng of each ancillary protein cRNA (*Asu-RIC*-3, *Asu-UNC*-50, *Asu-UNC*-74 and *Xle-RIC*-3) in a total volume of 50 nL.

4.3.6 Drug applications

All drugs used, except tribendimidine and derquantel, were purchased from Sigma Aldrich (St Louis, MO, USA). The drugs were solubilized in recording solution or DMSO (final working concentration did not exceed 0.1%). Derquantel and tribendimidine were a generous gift from Zoetis (Kalamazoo, MI, USA) and Prof Shu Hua Xiao (National Institute of Parasitic Diseases, China) respectively.

Agonists of interest were used at a final concentration of 100 μM except tribendimidine (30 μM) due to solubility issues. In all experiments, 100 μM acetylcholine (ACh) was applied first and all the responses were normalized to this control response. Each agonist was applied for 10s followed by 3 minutes perfusion with recording solution. The sequence for application of agonists for determining the rank order potency series was random and not predetermined. The concertation-response studies were conducted by application of the drug in ascending order of concentrations in order to minimize any potential desensitization by high concentrations. In each experiment the drug was applied for 10s followed by 3 minutes wash off with the recording solution.

All the antagonists in our study were used at a final concentration of 10 μM. For generating rank order potency series, a control application of 100 μM ACh (30 s) was first applied followed by 3 minutes wash off. Thereafter, 100 μM acetylcholine was applied for 10 s, immediately followed by 10 s application of the antagonist in the continued presence of 100 μM ACh and then a final 10 s application of 100 μM ACh. At least 3 min drug wash off interval was allowed between applications in order to minimize desensitization. Note that, due to short time of drug application in this protocol, it is possible to underestimate the potency of an antagonists.

4.3.7 Data and statistical analysis

Clampfit 10.3 (Molecular Devices, Sunnyvale, CA, USA) and GraphPad Prism 8.0 (GraphPad Software Inc., La Jolla, CA, USA) were used to analyze data. The peak currents in response to the applied agonists were measured and normalized to the control current (100 μM acetylcholine). The results were expressed as mean ± SEM. The Hill equation was used to analyze the concentration-response relationships by fitting log concentration-response data points as described in (Boulin et al. 2008). Desensitization kinetics in response to the agonists were fitted using a single exponential decay fit:

$$f(t) = \sum_{i=1}^{n} A_i e^{-t/\tau i+C}$$

Where n is the number of components, A is the amplitude, t is time, τ is the time constant, and C is the constant y-offset for each i component. The mean % inhibition produced by the antagonists on currents elicited by 100 μM acetylcholine were calculated using the equation previously described (Zheng et al. 2016). One-way ANOVA and extra sum of squares F-tests were used to test statistical differences between desensitization rate and pEC_{50} respectively. The significance levels were set to $P < 0.05$.

4.4 Results

4.4.1 Sequence comparison of *Aca*-ACR-16 with *Asu*-ACR-16

We were able to identify the putative complete coding sequence of the homologue of ACR-16 in the translated *Ancylostoma caninum* genome (Gene ID: ANCCAN_01899) by using the *Asu*-ACR-16 protein sequence (GenBank: KP756901) as a query in a BLASTP search in the nematode protein database, WormBase Parasite. When amplified, the *Aca*-ACR-16 was shorter than ANCCAN_01899 and lacked 19 amino acids between

Figure 4.0 Amino acid sequence alignment of *Aca*-ACR-16 and *Asu*-ACR-16. The signal peptide (light brown), ligand binding loops (A to F; maroon) transmembrane regions TM1-4 (blue), and cys-loop (green) are indicated. The adjacent cysteines (in the Y-X-C-C motif) in Loop-C are indicated in the black box. The negatively charge amino acids (E: Glutamic acid and D: Aspartic acid) flanking the TM2 domain are highlighted in orange. Note: The sequence of *Aca*-ACR-16 amplified from *A. caninum* larval total RNA is shorter than the WormBase sequence ANCCAN_01899 and lacks 19 amino acids (KVKEPNLFGPWENFHGDLF) between the cys-loop and Loop-B. These amino acid residues are also lacking in the *A. suum* ACR-16 homologue.

the cys-loop and loop-B; these amino acids are also missing in the published sequence of *Asu*-ACR-16. *Aca*-ACR-16 has all the structural characteristics of a nicotinic acetylcholine receptor subunit (Figure 4.0): a large extracellular NH_2-terminal domain of

~200 amino acids involved in correct nAChR assembly, a Cys-loop motif separated by 13 intervening amino acids, four transmembrane (TM) domains that form the ion-conducting pore, a cytoplasmic domain inserted between TM3 and TM4, six loops (A-F) and most importantly the presence of vicinal cysteines (a Y-x-C-C motif) in the C-loop making it an alpha subunit. Figure 4.0 shows the protein sequence alignment of *Aca*-ACR-16 with *Asu*-ACR-16. The two worm species belong to different clades of nematode, but their amino acid residues were highly conserved with an identity of 78% (87% similarity). There was a lack of conservation in *Aca*-ACR-16 loops E and F (involved in agonist binding) which encouraged us to characterize the pharmacology of the ion channel.

4.4.2 The ancillary factor RIC-3 is required for the functional expression of *Aca*-ACR-16

For the heterologous expression of the *Aca*-ACR-16, we expressed the subunit protein cRNA with different ancillary proteins (RIC-3, UNC-50 and UNC-74 from *A. suum* and RIC-3 from *X. laevis;* Figure 4.1). None of the combinations, except *Aca*-ACR-16 with *Asu*-ric-3, gave robust responses to control 100 μM ACh. In order to optimize the expressed receptor, we varied the amount of cRNA of *Aca*-ACR-16 (25-50 ng) and *Asu*-ric-3 (15-25 ng). We obtained the largest response from oocytes injected with 50 ng *Aca*-ACR-16 and 25 ng Asu-ric-3 and this mix was used for all subsequent recordings.

4.4.3 *Aca*-ACR-16 forms 3-bromocytisine sensitive nAChR

We tested a selection of nicotinic agonists including cholinergic anthelmintics on the expressed *A. caninum* ACR-16 ligand gated ion channel. Figure 4.2A shows the rank order potency series for the agonists on the expressed *Aca*-ACR-16 receptor. 3-Bromocytisine was the most potent agonist (>130% of the acetylcholine current).

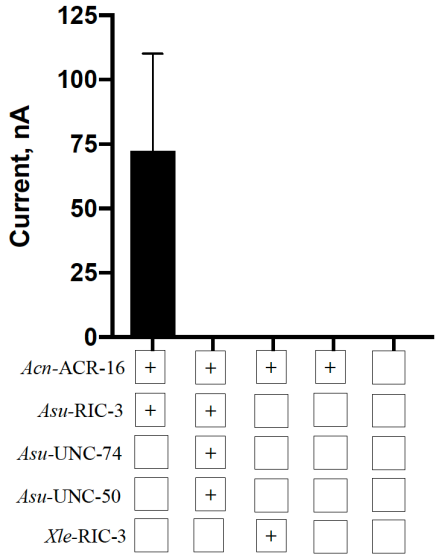

Figure 4.1 Bar chart showing the effects of ancillary proteins on the expression of *Aca*-ACR-16 nAChR. The receptor was able to express functionally only when co-injected with *Asu*-RIC-3.

Epibatidine, cytisine, nicotine and DMPP also activated the receptor. Interestingly the cholinergic anthelmintics including levamisole, oxantel, pyrantel, morantel, bephenium, and tribendimidine were not active on the expressed nAChR. The rank order potency series on *Aca*-ACR-16 when normalized to the control 100 µM acetylcholine current was: 3-bromocytisine > ACh > epibatidine > cytisine > nicotine > DMPP >> levamisole = oxantel = pyrantel = morantel = choline = bephenium = tribendimidine. None of cholinomimetic anthelmintics currently used in the field activated the homomeric receptor which shows that this channel is distinct from the other somatic nAChRs of nematodes.

4.4.4 Comparative pharmacology of acetylcholine and 3-bromocytisine

Figure 4.2B shows the concentration-response relationships of acetylcholine and 3-bromocytisine for the *A. caninum* homomeric channel. The sigmoidal plots were constructed by application of drugs in ascending order (0.3-300 µM depending on the agonist). 3-Bromocytisine (EC_{50} = 1.5 µM) was ~33 times more potent than acetylcholine

(EC_{50} = 50.0 µM) on the receptor. The curves for both the nicotinic agonists were steep with the hillslope (n_H) values greater than 1. This suggests that the ligands are binding to more than one site in the receptor and exhibit positive cooperativity as expected of a homomeric ligand-gated ion channel.

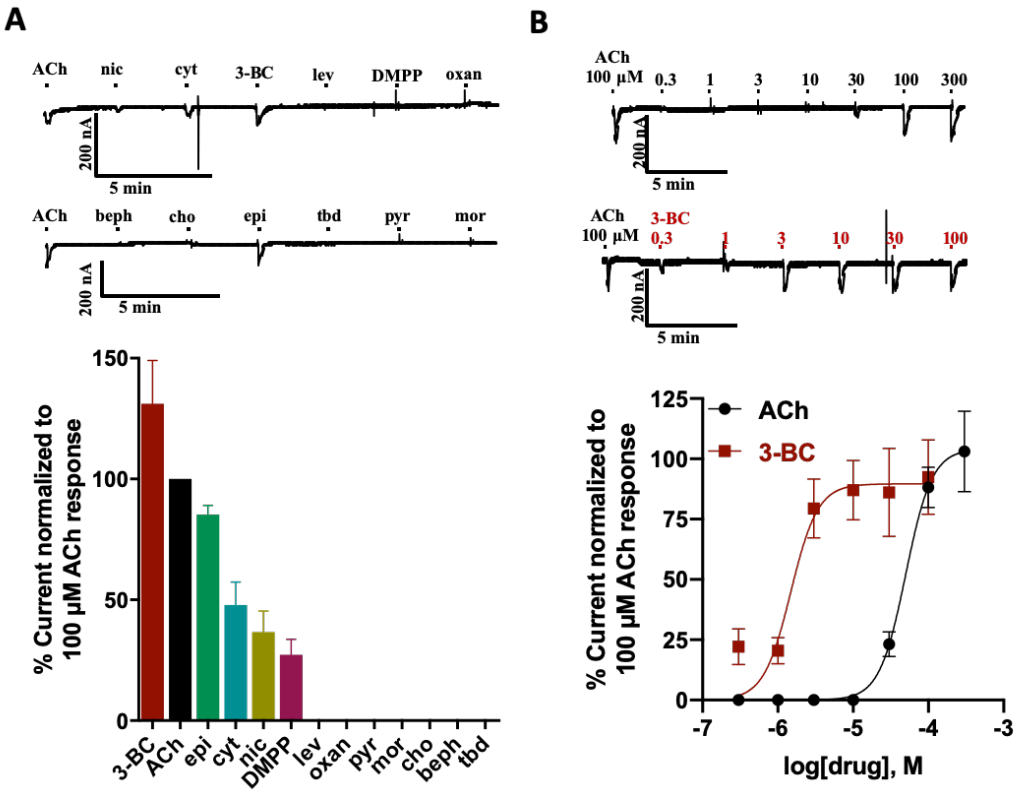

Figure 4.2 The effects of nAChR agonists and antiparasitic drugs on the *Aca*-ACh-16 receptor. A. Bar graph (mean ± SEM, %, n = 4) along with sample traces showing the effect of agonists and anthelmintics on the nAChR. The rank order potency series when normalized to the control 100 µM ACh current was as follows: 3-bromocytisine (3-BC; 131.0 ± 18.0) > ACh (100.0 ± 0.0) > epibatidine (epi; 85.0 ± 4.0) > cytisine (cyt; 48.0 ± 9.5) > nicotine (nic; 37.0 ± 8.7) > DMPP (Dimethyl-4-phenylpiperazinium; 27.0 ± 6.4) >>> levamisole (lev; 0.0 ± 0.0) = oxantel (oxan; 0.0 ± 0.0) = pyrantel (pyr; 0.0 ± 0.0) = morantel (mor; 0.0 ± 0.0) = choline (cho; 0.0 ± 0.0) = bephenium (beph; 0.0 ± 0.0) = tribendimidine (tbd; 0.0 ± 0.0). B. Sample traces and concentration-response relationships of 3-Bromocytisine and ACh for *Ancylostoma caninum* ACR-16. The pEC_{50} and hill slope (n_H) values, expressed as mean ± SEM, were respectively 4.3 ± 0.0 and 2.5 ± 0.3 for ACh (n=6); 5.0 ± 0.1 and 2.4 ± 0.7 for 3-BC (n=6).

4.4.5 *Aca*-ACR-16 desensitization

Desensitization is defined as decrease or loss of biological response following prolonged or repetitive stimulation. It is a common feature of many nAChRs including α-7 homomeric nAChRs (Giniatullin, Nistri, and Yakel 2005; Picciotto et al. 2008; Quick and Lester 2002). In the case of *A. suum* ACR-16, all the potent agonists exhibited desensitization (Abongwa, Buxton, et al. 2016). We observed a similar trend characterized by peak and then waning current responses observed during maintained (10 s) agonist applications with *Aca*-ACR-16 as shown in Figure 4.3. The time constant for desensitization was highest for epibatidine and lowest for 3-bromocytisine. The mean time constants for desensitization rates ranged between 1.5 and 4.8 s for the *Aca*-ACR-16 and were less than the rates observed in the *Asu*-ACR-16 (Abongwa, Buxton, et al. 2016).

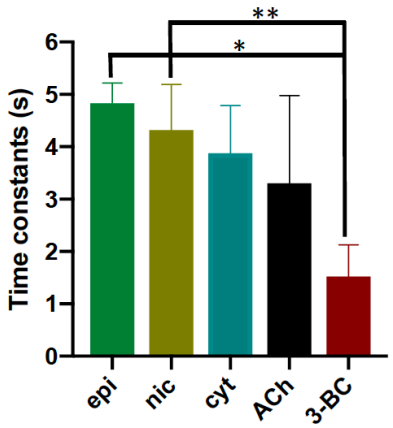

Figure 4.3 Bar graph showing desensitization time constants of the *Ancylostoma caninum* ACR-16 nAChR in response to agonists (100 µM, n=4). The rank order of time constants of desensitization (mean ± SEM, s) was as follows: epi (4.8 ± 0.2) > nic (4.3 ± 0.5) > cyt (3.9 ± 0.5) > ACh (3.3 ± 1.0) > 3-BC (1.5 ± 0.3). *$P < 0.05$, **$P < 0.01$; significantly different as indicated; Tukey's multiple comparison test.

4.4.6. Antagonist pharmacology

Six nAChR antagonists (10 µM each) were tested on the expressed cation selective *Aca*-ACR-16 channel. The antagonists were d-tubocurarine (dTC), mecamylamine,

dihydro-β-erythroidine (DHβE), derquantel, hexamethonium, and α-bungarotoxin (α-BTX). α-BTX produced least inhibition of the acetylcholine mediated current while d-TC and mecamylamine produced ~100% inhibition of the control current. DHβE, a selective antagonist for $\alpha_4\beta_2$ receptors (Levin 2002), interestingly also produced almost complete inhibition of acetylcholine currents. The complete rank order potency for antagonists (Figure 4.4) was: dTC ≈ mecamylamine ≈ DHβE > derquantel > hexamethonium > α-BTX.

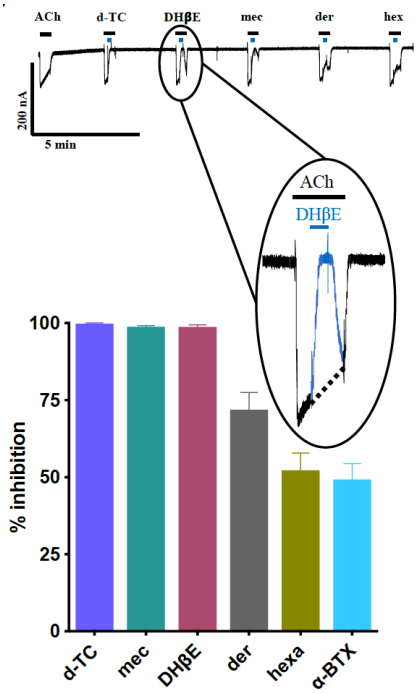

Figure 4.4 Effects of selected nAChR antagonists on the *Aca*-ACR-16. Sample trace and bar chart showing inhibition (mean ± SEM, %) of acetylcholine mediated currents by the selected antagonists (10 µM). d-tubocurarine (d-TC), mecamylamine (mec) and dihydro-β-erythroidine (DhβE) produced almost complete inhibition of ACh mediated responses. Derquantel (der) and hexamethonium (hexa) produced moderate blockade of *Aca*-ACR-16 mediated ACh responses and α-BTX was the least potent antagonist. The rank order potency series for nAChR antagonists is as follows: d-TC (100.0 ± 0.1) ≈ mec (98.8 ± 0.6) ≈ DHβE (98.8 ± 0.4) > der (72.0 ± 5.6) > hexa (52.2 ± 5.6) > α-BTX (49.3 ± 5.2). Inset: magnified view of current trace showing predicted acetylcholine response in the absence of DHβE (dotted line) and inhibition of acetylcholine mediated response in the presence of DHβE (highlighted in blue).

4.5 Discussion

4.5.1 Comparison of pharmacology of *Aca*-ACR-16 with homologues from other nematodes

In this study we have shown that ACR-16 from *Ancylostoma caninum*, a Clade V nematode and model for human hookworm infections, expresses as a homomeric channel in *Xenopus* oocytes. Abongwa, Buxton, et al. (2016) successfully recapitulated and characterized the ACR-16 homologue from *Ascaris suum*, a clade III gastrointestinal parasite. Ballivet et al. (1996) characterized the pharmacology of ACR-16 nAChR from *Caenorhabditis elegans*, a clade V free-living nematode. Similar to the *A. suum* and *C. elegans* channel, *Aca*-ACR-16 was not sensitive to many of the currently used cholinomimetic anthelmintics including levamisole, pyrantel and tribendimidine. However, the *A. caninum* nAChR was most sensitive to 3-bromocytisine while nicotine was the most potent agonist for the *A. suum* and *C. elegans* ACR-16 receptors. The acetylcholine concentration-response curve for *Aca*-ACR-16 (n_H = 2.5 ± 0.3) had comparable slope factor to *Cel*-ACR-16 (n_H = 2.1) but was shallower in comparison to the *Asu*-ACR-16 (n_H = 3.9 ± 0.3). This may account for the higher sensitivity to acetylcholine for the *A. suum* α nAChR (EC_{50} = 5.9 μM) and the similar sensitivity to the agonist for *A. caninum* (EC_{50} = 50.0 μM) and *C. elegans* (EC_{50} = 55.4 μM) ACR-16 receptors. In terms of antagonist pharmacology, the *A. caninum* cation channel was moderately inhibited by α-BTX (mean ± SEM = 49.3 ± 5. 2 %) while *Asu*-ACR-16 and *Cel*-ACR-16 nAChRs were nearly insensitive. DhβE produced complete inhibition of acetylcholine mediated responses on the *Aca*-ACR-16 and *Cel*-ACR-16 nAChRs while the *A. suum* homologue was only moderately inhibited. Similar to *Asu*-ACR-16, the *Aca*-

ACR-16 receptor was highly sensitive to mecamylamine and d-TC; moderately sensitive to derquantel and hexamethonium. The protein sequence of the ACR-16 homologues from all the nematode parasites is highly conserved; *Aca*-ACR-16 shares 78 % identity with *A. suum* and *C. elegans* homologues. There are variable amino acids residues in the loops E and F which can account for differences in the pharmacological properties (Corringer, Novère, and Changeux 2000).

4.5.2 Consideration of the *Aca*-ACR-16 as a drug target

Hookworm infections affect approximately 500 million people globally, with 5.1 billion at risk of acquiring infections (Global Burden of Disease Study 2015; Pullan and Brooker 2012). Despite decades of strong research efforts and identification of promising candidate antigens, there are still no commercially available vaccines for human hookworm infections. Consequently, identification of novel drug targets and development of associated therapeutic agents is a logical approach for future defense against these infections. In parasitic nematodes nicotinic acetylcholine receptors are required for various physiological functions. These ligand-gated ion channels are targets of important cholinergic antinematodal drugs such as levamisole and pyrantel. Recently introduced "novel" anthelmintics including tribendimidine and derquantel also target nematode nAChRs (Abongwa, Martin, and Robertson 2017; Wolstenholme 2011). *acr-16* encodes for nicotine-sensitive nAChRs that are expressed in body wall muscles in *C. elegans* and contribute to the fast synaptic cholinergic neurotransmission in these muscles (Francis et al. 2005; Touroutine et al. 2005; Richmond and Jorgensen 1999). The *unc-63*:*acr-16* double mutants in *C. elegans* exhibit locomotor defects which are more severe than either *unc-63* or *acr-16* alone (Touroutine et al. 2005). Similarly, *unc-29*:*acr-16*

double mutants also display far greater movement impairment than either *unc-29* or *acr-16* single mutants (Li et al. 2014). This demonstrates that ACR-16 in combination with UNC-63 and UNC-29, components of levamisole-sensitive nAChR, contributes to locomotor behavior in the worms. In *Brugia malayi*, a clade V worm, knockdown of *acr-16:acr-26* had no effect on motility (Verma et al. 2017) possibly suggesting a different physiological function of the ACR-16 homologue in the filarial worm. In *A. suum,* the ACR-16 homologue was detected in the ovijector and digestive tract tissues in addition to the body wall muscles. It is plausible that the ACR-16 nAChRs not only regulates neurotransmission in *A. suum* but also serve other tissue-related functions including reproduction and digestion. The ACR-16 homologue from *A. suum* has been suggested as a drug target (Abongwa, Buxton, et al. 2016). We have successfully reconstituted a fully functional homomeric nAChR, ACR-16, in the *Xenopus oocyte* expression system from *A. caninum*, a model for human hookworm infections. The pharmacology of the receptor is distinct from the levamisole sensitive nematode receptors (Martin et al. 2012; Boulin et al. 2008; Richmond and Jorgensen 1999). The *A. caninum* ACR-16 homologue also displayed some pharmacological differences from *Asu*-ACR-16. Benzimidazoles are the commonly used antiparasitic drugs for treatment of hookworm infections but there have been multiple reports of resistance in veterinary medicine (Kaplan 2004; Wolstenholme et al. 2004) and decreased cure rates in humans (Geerts and Gryseels 2000; De Clercq et al. 1997; Keiser and Utzinger 2008; Conder and Campbell 1995). There is a need for new drugs against hookworms and ACR-16 may be a valid target site with the potential to circumvent existing drug resistance.

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4.7 Conflict of interest

The authors declare that this work has no conflict of interest.

4.8 References

Abongwa, M., S. K. Buxton, E. Courtot, C. L. Charvet, C. Neveu, C. J. McCoy, S. Verma, A. P. Robertson, and R. J. Martin. 2016. 'Pharmacological profile of Ascaris suum ACR-16, a new homomeric nicotinic acetylcholine receptor widely distributed in Ascaris tissues', *Br J Pharmacol*, 173: 2463-77.

Abongwa, M., R. J. Martin, and A. P. Robertson. 2017. 'A Brief Review on the Mode of Action of Antinematodal Drugs', *Acta Vet (Beogr)*, 67: 137-52.

Aceves, J., D. Erlij, and R. Martínez-Marañón. 1970. 'The mechanism of the paralysing action of tetramisole on Ascaris somatic muscle', *British journal of pharmacology*, 38: 602-07.

Albonico, M., Q. Bickle, M. Ramsan, A. Montresor, L. Savioli, and M. Taylor. 2003. 'Efficacy of mebendazole and levamisole alone or in combination against intestinal nematode infections after repeated targeted mebendazole treatment in Zanzibar', *Bull World Health Organ*, 81: 343-52.

Albonico, M., P. G. Smith, E. Ercole, A. Hall, H. M. Chwaya, K. S. Alawi, and L. Savioli. 1995. 'Rate of reinfection with intestinal nematodes after treatment of children with mebendazole or albendazole in a highly endemic area', *Trans R Soc Trop Med Hyg*, 89: 538-41.

Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. 'Gapped BLAST and PSI-BLAST: a new generation of protein database search programs', *Nucleic Acids Res*, 25: 3389-402.

Aubry, M. L., P. Cowell, M. J. Davey, and S. Shevde. 1970. 'Aspects of the pharmacology of a new anthelmintic: pyrantel', *Br J Pharmacol*, 38: 332-44.

Ballivet, Marc, Christine Alliod, Sonia Bertrand, and Daniel Bertrand. 1996. 'Nicotinic Acetylcholine Receptors in the NematodeCaenorhabditis elegans', *Journal of Molecular Biology*, 258: 261-69.

Bartsch, S. M., P. J. Hotez, L. Asti, K. M. Zapf, M. E. Bottazzi, D. J. Diemert, and B. Y. Lee. 2016. 'The Global Economic and Health Burden of Human Hookworm Infection', *PLoS Negl Trop Dis*, 10: e0004922.

Bennett, A., and H. Guyatt. 2000. 'Reducing intestinal nematode infection: efficacy of albendazole and mebendazole', *Parasitol Today*, 16: 71-4.

Bethony, J., S. Brooker, M. Albonico, S. M. Geiger, A. Loukas, D. Diemert, and P. J. Hotez. 2006. 'Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm', *Lancet*, 367: 1521-32.

Blaxter, Mark. 2000. 'Genes and genomes of Necator americanus and related hookworms', *International Journal for Parasitology*, 30: 347-55.

Boulin, T., M. Gielen, J. E. Richmond, D. C. Williams, P. Paoletti, and J. L. Bessereau. 2008. 'Eight genes are required for functional reconstitution of the Caenorhabditis elegans levamisole-sensitive acetylcholine receptor', *Proc Natl Acad Sci U S A*, 105: 18590-5.

Bowman, Dwight D., Susan P. Montgomery, Anne M. Zajac, Mark L. Eberhard, and Kevin R. Kazacos. 2010. 'Hookworms of dogs and cats as agents of cutaneous larva migrans', *Trends in Parasitology*, 26: 162-67.

Brooker, S., P. J. Hotez, and D. A. Bundy. 2008. 'Hookworm-related anaemia among pregnant women: a systematic review', *PLoS Negl Trop Dis*, 2: e291.

Campbell, S. J., N. K. Biritwum, G. Woods, Y. Velleman, F. Fleming, and J. R. Stothard. 2018. 'Tailoring Water, Sanitation, and Hygiene (WASH) Targets for Soil-Transmitted Helminthiasis and Schistosomiasis Control', *Trends Parasitol*, 34: 53-63.

Choudhary, Shivani, Djordje S. Marjianović, Colin R. Wong, Xiaoyu Zhang, Melanie Abongwa, Joel R. Coats, Saša M. Trailović, Richard J. Martin, and Alan P. Robertson. 2019. 'Menthol acts as a positive allosteric modulator on nematode levamisole sensitive nicotinic acetylcholine receptors', *International Journal for Parasitology: Drugs and Drug Resistance*, 9: 44-53.

Conder, G. A., and W. C. Campbell. 1995. 'Chemotherapy of nematode infections of veterinary importance, with special reference to drug resistance', *Adv Parasitol*, 35: 1-84.

Corringer, P. J., N. Le Novere, and J. P. Changeux. 2000. 'Nicotinic receptors at the amino acid level', *Annu Rev Pharmacol Toxicol*, 40: 431-58.

De Clercq, D., M. Sacko, J. Behnke, F. Gilbert, P. Dorny, and J. Vercruysse. 1997a. 'Failure of Mebendazole in Treatment of Human Hookworm Infections in the Southern Region of Mali', *The American Journal of Tropical Medicine and Hygiene*, 57: 25-30.

———. 1997b. 'Failure of mebendazole in treatment of human hookworm infections in the southern region of Mali', *Am J Trop Med Hyg*, 57: 25-30.

de Silva, N. R., S. Brooker, P. J. Hotez, A. Montresor, D. Engels, and L. Savioli. 2003. 'Soil-transmitted helminth infections: updating the global picture', *Trends Parasitol*, 19: 547-51.

Dias, Sílvia Regina Costa, Denílson Eduardo Silva Cunha, Sydnei Magno da Silva, Hudson Andrade dos Santos, Ricardo Toshio Fujiwara, and Élida Mara Leite Rabelo. 2013. 'Evaluation of parasitological and immunological aspects of acute infection by Ancylostoma caninum and Ancylostoma braziliense in mixed-breed dogs', *Parasitology Research*, 112: 2151-57.

Diemert, D. J., J. M. Bethony, and P. J. Hotez. 2008. 'Hookworm vaccines', *Clin Infect Dis*, 46: 282-8.

Epe, C. 2009. 'Intestinal nematodes: biology and control', *Vet Clin North Am Small Anim Pract*, 39: 1091-107, vi-vii.

Flohr, C., L. N. Tuyen, S. Lewis, T. T. Minh, J. Campbell, J. Britton, H. Williams, T. T. Hien, J. Farrar, and R. J. Quinnell. 2007. 'Low efficacy of mebendazole against hookworm in Vietnam: two randomized controlled trials', *Am J Trop Med Hyg*, 76: 732-6.

Francis, Michael M., Susan P. Evans, Michael Jensen, David M. Madsen, Joel Mancuso, Kenneth R. Norman, and Andres Villu Maricq. 2005. 'The Ror Receptor Tyrosine Kinase CAM-1 Is Required for ACR-16-Mediated Synaptic Transmission at the C. elegans Neuromuscular Junction', *Neuron*, 46: 581-94.

Geerts, S., and B. Gryseels. 2000. 'Drug resistance in human helminths: current situation and lessons from livestock', *Clin Microbiol Rev*, 13: 207-22.

Gibson, D. G., L. Young, R. Y. Chuang, J. C. Venter, C. A. Hutchison, 3rd, and H. O. Smith. 2009. 'Enzymatic assembly of DNA molecules up to several hundred kilobases', *Nat Methods*, 6: 343-5.

Giniatullin, R., A. Nistri, and J. L. Yakel. 2005. 'Desensitization of nicotinic ACh receptors: shaping cholinergic signaling', *Trends Neurosci*, 28: 371-8.

Global Burden of Disease Study, Collaborators. 2015. 'Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013', *Lancet*, 386: 743-800.

Guyatt, H. L., S. Brooker, N. Peshu, and C. E. Shulman. 2000. 'Hookworm and anaemia prevalence', *Lancet*, 356: 2101.

Harrow, Ian D., and Kenneth A. F. Gration. 1985. 'Mode of action of the anthelmintics morantel, pyrantel and levamisole on muscle cell membrane of the nematode Ascaris suum', *Pesticide Science*, 16: 662-72.

Hewitson, J. P., and R. M. Maizels. 2014. 'Vaccination against helminth parasite infections', *Expert Rev Vaccines*, 13: 473-87.

Hofmann, K., and W. Stoffel. 1993. 'TMbase - A database of membrane spanning proteins segments', *Biol. Chem. Hoppe-Seyler*, 374.

Hotez, P. 2008. 'Hookworm and poverty', *Ann N Y Acad Sci*, 1136: 38-44.

Hotez, P. J., M. Alvarado, M. G. Basanez, I. Bolliger, R. Bourne, M. Boussinesq, S. J. Brooker, A. S. Brown, G. Buckle, C. M. Budke, H. Carabin, L. E. Coffeng, E. M. Fevre, T. Furst, Y. A. Halasa, R. Jasrasaria, N. E. Johns, J. Keiser, C. H. King, R. Lozano, M. E. Murdoch, S. O'Hanlon, S. D. Pion, R. L. Pullan, K. D. Ramaiah, T. Roberts, D. S. Shepard, J. L. Smith, W. A. Stolk, E. A. Undurraga, J. Utzinger, M. Wang, C. J. Murray, and M. Naghavi. 2014. 'The global burden of disease study 2010: interpretation and implications for the neglected tropical diseases', *PLoS Negl Trop Dis*, 8: e2865.

Hotez, P. J., and D. I. Pritchard. 1995. 'Hookworm infection', *Scientific American*, 272: 68.

Jones, Andrew K., Paul Davis, Jonathan Hodgkin, and David B. Sattelle. 2007. 'The nicotinic acetylcholine receptor gene family of the nematode Caenorhabditis elegans: an update on nomenclature', *Invertebrate Neuroscience*, 7: 129-31.

Kaminsky, R., P. Ducray, M. Jung, R. Clover, L. Rufener, J. Bouvier, S. S. Weber, A. Wenger, S. Wieland-Berghausen, T. Goebel, N. Gauvry, F. Pautrat, T. Skripsky, O. Froelich, C. Komoin-Oka, B. Westlund, A. Sluder, and P. Maser. 2008. 'A new class of anthelmintics effective against drug-resistant nematodes', *Nature*, 452: 176-80.

Kaplan, Ray M. 2004. 'Drug resistance in nematodes of veterinary importance: a status report', *Trends in Parasitology*, 20: 477-81.

Keiser, J., and J. Utzinger. 2008. 'Efficacy of current drugs against soil-transmitted helminth infections: systematic review and meta-analysis', *JAMA*, 299: 1937-48.

Knopp, Stefanie, Peter Steinmann, Jennifer Keiser, and Jürg Utzinger. 2012. 'Nematode Infections: Soil-Transmitted Helminths and Trichinella', *Infectious Disease Clinics of North America*, 26: 341-58.

Krücken, Jürgen, Kira Fraundorfer, Jean Claude Mugisha, Sabrina Ramünke, Kevin C. Sifft, Dominik Geus, Felix Habarugira, Jules Ndoli, Augustin Sendegeya, Caritas

Mukampunga, Claude Bayingana, Toni Aebischer, Janina Demeler, Jean Bosco Gahutu, Frank P. Mockenhaupt, and Georg von Samson-Himmelstjerna. 2017. 'Reduced efficacy of albendazole against Ascaris lumbricoides in Rwandan schoolchildren', *International journal for parasitology. Drugs and drug resistance*, 7: 262-71.

Landmann, J. K., and P. Prociv. 2003. 'Experimental human infection with the dog hookworm, Ancylostoma caninum', *Med J Aust*, 178: 69-71.

Levin, E. D. 2002. 'Nicotinic receptor subtypes and cognitive function', *J Neurobiol*, 53: 633-40.

Li, Zhaoyu, Jie Liu, Maohua Zheng, and X. Z. Shawn Xu. 2014. 'Encoding of Both Analog- and Digital-like Behavioral Outputs by One C. elegans Interneuron', *Cell*, 159: 751-65.

Loukas, Alex, Peter J. Hotez, David Diemert, Maria Yazdanbakhsh, James S. McCarthy, Rodrigo Correa-Oliveira, John Croese, and Jeffrey M. Bethony. 2016. 'Hookworm infection', *Nature Reviews Disease Primers*, 2: 16088.

Martin, R. J., S. Verma, M. Levandoski, C. L. Clark, H. Qian, M. Stewart, and A. P. Robertson. 2005. 'Drug resistance and neurotransmitter receptors of nematodes: recent studies on the mode of action of levamisole', *Parasitology*, 131 Suppl: S71-84.

Martin, Richard J., Alan P. Robertson, Samuel K. Buxton, Robin N. Beech, Claude L. Charvet, and Cédric Neveu. 2012. 'Levamisole receptors: a second awakening', *Trends in Parasitology*, 28: 289-96.

Menzies, S. K., A. Rodriguez, M. Chico, C. Sandoval, N. Broncano, I. Guadalupe, and P. J. Cooper. 2014. 'Risk factors for soil-transmitted helminth infections during the first 3 years of life in the tropics; findings from a birth cohort', *PLoS Negl Trop Dis*, 8: e2718.

Nemzek, Jean A., Patrick A. Lester, A. Marissa Wolfe, Robert C. Dysko, and Daniel D. Myers. 2015. 'Chapter 12 - Biology and Diseases of Dogs.' in James G. Fox, Lynn C. Anderson, Glen M. Otto, Kathleen R. Pritchett-Corning and Mark T. Whary (eds.), *Laboratory Animal Medicine (Third Edition)* (Academic Press: Boston).

Petersen, T. N., S. Brunak, G. von Heijne, and H. Nielsen. 2011. 'SignalP 4.0: discriminating signal peptides from transmembrane regions', *Nat Methods*, 8: 785-6.

Picciotto, M. R., N. A. Addy, Y. S. Mineur, and D. H. Brunzell. 2008. 'It is not "either/or": activation and desensitization of nicotinic acetylcholine receptors both contribute to behaviors related to nicotine addiction and mood', *Prog Neurobiol*, 84: 329-42.

Prociv, P., and J. Croese. 1996. 'Human enteric infection with Ancylostoma caninum: hookworms reappraised in the light of a "new" zoonosis', *Acta Trop*, 62: 23-44.

Pullan, R. L., and S. J. Brooker. 2012. 'The global limits and population at risk of soil-transmitted helminth infections in 2010', *Parasit Vectors*, 5: 81.

Pullan, R. L., J. L. Smith, R. Jasrasaria, and S. J. Brooker. 2014. 'Global numbers of infection and disease burden of soil transmitted helminth infections in 2010', *Parasit Vectors*, 7: 37.

Quick, M. W., and R. A. Lester. 2002. 'Desensitization of neuronal nicotinic receptors', *J Neurobiol*, 53: 457-78.

Reynoldson, James A., Jerzy M. Behnke, Louise J. Pallant, Marion G. Macnish, Francis Gilbert, S. Giles, R. J. Spargo, and R. C. Andrew Thompson. 1997. 'Failure of pyrantel in treatment of human hookworm infections (Ancylostoma duodenale) in the Kimberley region of North West Australia', *Acta Tropica*, 68: 301-12.

Richmond, J. E., and E. M. Jorgensen. 1999. 'One GABA and two acetylcholine receptors function at the C. elegans neuromuscular junction', *Nat Neurosci*, 2: 791-7.

Sievers, F., A. Wilm, D. Dineen, T. J. Gibson, K. Karplus, W. Li, R. Lopez, H. McWilliam, M. Remmert, J. Soding, J. D. Thompson, and D. G. Higgins. 2011. 'Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega', *Mol Syst Biol*, 7: 539.

Thompson, A. J., H. A. Lester, and S. C. Lummis. 2010. 'The structural basis of function in Cys-loop receptors', *Q Rev Biophys*, 43: 449-99.

Touroutine, Denis, Rebecca M. Fox, Stephen E. Von Stetina, Anna Burdina, David M. Miller, and Janet E. Richmond. 2005. 'acr-16 Encodes an Essential Subunit of the Levamisole-resistant Nicotinic Receptor at the Caenorhabditis elegans Neuromuscular Junction', *Journal of Biological Chemistry*, 280: 27013-21.

Traversa, D. 2012. 'Pet roundworms and hookworms: a continuing need for global worming', *Parasit Vectors*, 5: 91.

Verma, S., S. S. Kashyap, A. P. Robertson, and R. J. Martin. 2017. 'Functional genomics in Brugia malayi reveal diverse muscle nAChRs and differences between cholinergic anthelmintics', *Proc Natl Acad Sci U S A*, 114: 5539-44.

WHO. 2015. *Investing to overcome the global impact of neglected tropical diseases: third WHO report on neglected diseases 2015. WHO Press, World Health Organization, Geneva* (World Health Organisation, Geneva).

Wolstenholme, A. J. 2011. 'Ion channels and receptor as targets for the control of parasitic nematodes', *Int J Parasitol Drugs Drug Resist*, 1: 2-13.

Wolstenholme, Adrian J., Ian Fairweather, Roger Prichard, Georg von Samson-Himmelstjerna, and Nicholas C. Sangster. 2004. 'Drug resistance in veterinary helminths', *Trends in Parasitology*, 20: 469-76.

Zheng, F., A. P. Robertson, M. Abongwa, E. W. Yu, and R. J. Martin. 2016. 'The Ascaris suum nicotinic receptor, ACR-16, as a drug target: Four novel negative allosteric modulators from virtual screening', *Int J Parasitol Drugs Drug Resist*, 6: 60-73.

CHAPTER 5. MENTHOL ACTS AS A POSITIVE ALLOSTERIC MODULATOR ON NEMATODE LEVAMISOLE SENSITIVE NICOTINIC ACETYLCHOLINE RECEPTORS

A paper published in the *International Journal for Parasitology: Drugs and Drug Resistance* (2019)[1]

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5.1 Abstract

The ongoing and widespread emergence of resistance to the existing anti-nematodal pharmacopeia has made it imperative to develop new anthelminthic agents. Historically, plants have been important sources of therapeutic compounds and offer an alternative to synthetic drugs. Monoterpenoids are phytochemicals that have been shown to produce acute toxic effects in insects and nematodes. Previous studies have shown nicotinic acetylcholine receptors (nAChRs) to be possible targets for naturally occurring plant metabolites such as carvacrol and carveol. In this study we examined the effects of monoterpenoid compounds on a levamisole sensitive nAChR from *Oesophagostomum dentatum* and a nicotine sensitive nAChR from *Ascaris suum*. We expressed the receptors in *Xenopus laevis* oocytes and used two-electrode voltage-clamp electrophysiology technique to characterize the effect of various compounds on these cys-loop receptors. At

100 μM the majority of these compounds acted as antagonists. Interestingly, further experiments revealed that both 0.1 μM and 10 μM menthol potentiated acetylcholine and levamisole responses in the levamisole sensitive receptor but not the nicotine sensitive receptor. We also investigated the effects of 0.1 μM menthol on the contractility of *A. suum* somatic muscle strips. Menthol produced significant potentiation of peak contractions at each concentration of acetylcholine. The positive allosteric modulatory effects of menthol in both *in vivo* and *in vitro* experiments suggest menthol as a promising candidate for combination therapy with cholinergic anthelmintics.

Keywords

Menthol, allosteric modulation, nAChR, monoterpenoid, nematode

5.2 Abbreviations

Asu, *Ascaris suum*; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester); cRNA, complementary RNA; DMSO, dimethyl sulfoxide; *Hco, Haemonchus contortus*; nAChR, nicotinic acetylcholine receptor; *Ode*, *Oesophagustomum dentatum*; PAM, positive allosteric modulator; STHs, soil transmitted helminths.

5.3 Introduction

Parasitic nematode infections are a threat to health and have significant socio-economic consequences. Among the different classes of nematodes, soil transmitted helminths (STHs) are major contributors to the global parasite burden. According to WHO (2018), more than 1.5 billion people, or 24% of the world's population, are

infected with at least one species of soil transmitted helminth, which mainly include *Ascaris lumbricoides*, *Trichuris trichiura* and hookworms (*Necator americanus* and *Ancylostoma duodenale*). These infections are most commonly found in tropical and sub-tropical countries due to suboptimal sanitation, lack of access to clean water and favorable climatic conditions for the parasites (Bethony et al. 2006; Brooker, Clements, and Bundy 2006; Savioli and Albonico 2004; Hotez et al. 2008). Although mortality is rare, high morbidity is a common feature of intestinal helminthiasis. The disease manifestations include weight loss, anemia, diarrhea, abdominal pain, malnutrition, general malaise, weakness, as well as impaired growth and physical development (Bethony et al. 2006). Children bear the highest burden of these infections which profoundly affect their physical growth, cognitive development and cause educational deficits (Brooker 2010; Stephenson, Latham, and Ottesen 2000; Alum, Rubino, and Ijaz 2010). STH infections such as *Ascariasis* have also been reported to exacerbate other diseases such as malaria, tuberculosis and HIV/AIDS (Fincham, Markus, and Adams 2003; Le Hesran et al. 2004). In addition, production losses caused by intestinal nematodes of livestock have adverse effects on nutritional status and the food economy especially in poverty-stricken regions (Morgan et al. 2013).

Effective vaccines are the most desirable approach but even after almost 30 years of dedicated research, a licensed vaccine against human parasites remains elusive. Current control strategies for treatment and prevention of helminth infections rely heavily on chemotherapy. Unfortunately, there are a limited number of classes of anthelmintic agents, which include benzimidazoles, macrocyclic lactones and nicotinic agonists (Abongwa, Martin, and Robertson 2017; Martin 1997; Robertson and Martin 2007).

Moreover, widespread and indiscriminate administration combined with genetic factors has led to the development of resistance in animals (Taman and Azab 2014; Albonico et al. 2003). There have also been reports of reduced therapeutic efficacy to anti-nematodal drugs in humans. De Clercq et al. (1997) and Flohr et al. (2007) reported poor efficacy of benzimidazoles against human hookworm infections. There is a need to focus research on drug discovery and development to overcome the issue of increasing resistance and reduced efficacy against existing drugs.

The use of phytotherapeutics for parasitic infections has emerged as an attractive alternative. Plants have dominated the human pharmacopoeia for thousands of years (Balick and Cox 1996; Raskin and Ripoll 2004; Newman and Cragg 2016). Many therapeutic products, including codeine, quinine, artemisinin, digitoxin, morphine, paclitaxel, galantamine, currently used in modern pharmacotherapy are of plant origin (Newman and Cragg 2016; Newman, Cragg, and Snader 2000; Butler 2004; Heinrich and Lee Teoh 2004; Pirttila et al. 2004; Samuelsson 2004; Petrovska 2012). Plant based therapeutic compounds are environment-friendly and exhibit a wide array of medicinal properties (Coats 1994; Hu and Coats 2008; Balunas and Kinghorn 2005; Harvey, Edrada-Ebel, and Quinn 2015; Dias, Urban, and Roessner 2012). Interestingly, an estimated four billion people living in the developing world rely on medicinal plants for their primary pharmaceutical care (WHO 2005). Herbal remedies in the form of complementary and alternative medicine (CAM) are also being widely embraced in many developed countries (Committee on the Use of Complementary 2005; Calapai 2008; Anquez-Traxler 2011). Among phytochemicals, essential oils and their active principles have been well documented for their broad range of functions including insecticidal and

nematocidal activities (Enan 2005; Panella et al. 2005; Camurca-Vasconcelos et al. 2007; Coskun et al. 2008; Macedo et al. 2009; Cetin et al. 2009). Historically, thymol (found in oil of thyme), santonin (derived from buds of the local *Artemisia* spp.) and ascaridol (isolated from Chenopodium oil) were used in the late nineteenth and early twentieth century for the treatment of ascarids and hookworms in humans (Ferrell 1914; Lamson and Ward 1932; Kaplan et al. 2014). Terpene and terpenoid biomolecules, particularly monoterpenes and sesquiterpenes, found in essential oils have been identified as promising anthelmintic compounds and are of great pharmaceutical interest (Lei, Leser, and Enan 2010; Pessoa et al. 2002; Squires et al. 2010; Jaradat et al. 2016; Katiki, Chagas, et al. 2011). Lei, Leser, and Enan (2010) reported *in vitro* nematocidal activity of thymol and carvacrol (major components of thyme and oregano essential oils) against *Caenorhabditis elegans* and *Ascaris suum*. Trailović et al. (2015) also demonstrated the potential anthelmintic effects of carvacrol in *A. suum*.

In the present study, we tested the activity of monoterpenoid compounds on two different nematode nAChR types: levamisole sensitive (L-type) nAChRs from *Oesophagostomum dentatum* and a nicotine sensitive (N-type) nAChR (ACR-16) from *A. suum*. The receptors were expressed in *Xenopus laevis* oocytes and we used two-electrode voltage-clamp to study the pharmacological effects of the compounds. We also investigated the effects of menthol on the contractility of *A. suum* somatic muscle strips. The focus of this study was to evaluate the effects produced by monoterpenoid compounds to identify potential alternatives to current chemotherapeutic agents.

5.4 Materials and Methods

5.4.1 cRNA preparation and *X. laevis* oocyte expression

All nAChR subunit and ancillary factor cRNAs from *O. dentatum* (*Ode-unc-29, Ode-unc-38, Ode-unc-63 and Ode-acr-8*), *A. suum* (*Asu-acr-16* and *Asu-ric-3*) and *Haemonchus contortus* (*Hco-ric-3, Hco-unc-50 and Hco-unc-74*) were prepared as previously described (Abongwa, Buxton, et al. 2016; Buxton et al. 2014). The receptors were expressed in defolliculated *X. laevis* oocytes purchased from Ecocyte Bioscience (Austin, Texas, USA). N-type (nicotine sensitive) nAChRs from *A. suum* were expressed by co-injecting 25 ng of *Asu-acr-16* with 5 ng of *Asu-ric-3* in a total volume of 50 nL in nuclease-free water. Heteromeric (levamisole sensitive) nAChRs from *O. dentatum* were expressed by co-injecting 1.8 ng of each subunit cRNA (*Ode-unc-29*, *Ode-unc-38*, *Ode-unc-68* and *Ode-acr-8*) with 1.8 ng of each *H. contortus* ancillary factor (*Hco-ric-3*, *Hco-unc-50* and *Hco-unc-74*) in a total volume of 36 nL in nuclease-free water. The injections were made in the animal pole of the oocytes using a nanoject II microinjector (Drummond Scientific, Broomall, PA, USA). The injected oocytes were separated into 96-well plates containing 200 µL incubation solution (100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2.2H_20$, 1 mM $MgCl_2.6H_20$, 5 mM HEPES, 2.5 mM Na pyruvate, 100 $U \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin, pH 7.5); each well contained one oocyte. The solution was changed daily to maintain oocytes in optimal conditions. The oocytes were incubated at 19 ºC for at 5-7 days to allow functional expression of the receptor.

5.4.2 Two-electrode voltage-clamp (TEVC) electrophysiology

We used two-electrode voltage-clamp electrophysiology to record the inward current generated by the activation of expressed receptors in *X. laevis* oocytes. The oocytes were incubated with 100 μM BAPTA-AM (an intracellular calcium chelator) for ~3 hours prior to recordings to prevent activation of endogenous calcium-activated chloride currents. The oocytes were clamped at -60mV for all the experiments with an Axoclamp 2B amplifier; all data were acquired on a desktop computer with Clampex 10.2 (Molecular Devices Inc., CA, USA). The microelectrodes used to impale oocytes were pulled using a Flaming/Brown horizontal micropipette puller (model P-97, Sutter Instruments Co., USA) and filled with 3 M KCl. The tips of the microelectrodes were carefully broken with a tissue paper to achieve a resistance of 2-5 MΩ in recording solution (100 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2 \cdot 2H_2O$ and 5 mM HEPES, pH 7.3). The low resistance allowed passage of large currents required to maintain adequate voltage-clamp of the oocyte. Oocytes were placed into a tiny groove of the narrow oocyte-recording chamber. The Digidata 1322A (Molecular Devices, CA, USA) was used to control the switches that controlled the perfusion of the chamber at a speed of ~6 mL/min. Un-injected oocytes served as the negative control.

5.4.3 *A. suum* muscle flap contraction measurements

The adult female *A. suum* worms were collected from the slaughterhouse at Surčin, Belgrade, Serbia and maintained at 32 °C in Locke's solution (NaCl 155 mM, KCl 5 mM, $CaCl_2$ 2 mM, $NaHCO_3$ 1.5 mM and glucose 5 mM). The Locke's solution was changed twice daily. Each batch of worms was used within 4 days of collection. For contraction studies, *A. suum* muscle flaps were prepared as described in Trailović et al. (2015). Briefly, an anterior part of the worm, 2-3 cm caudal to the head was dissected to

prepare a 1 cm muscle flap and the lateral line was removed from the edge of the flaps. Each muscle flap was attached to a force transducer in an experimental bath maintained at 37 °C, containing 20 mL Ascaris Perienteric Fluid Ringer (23 mM NaCl, 110 mM Na acetate, 24 mM KCl, 6 mM $CaCl_2$, 5 mM $MgCl_2$, 11 mM glucose, 5 mM HEPES, pH 7.6) and bubbled with room air. The isometric contractions were monitored on a PC computer using a BioSmart interface with eLAB software (EIUnit, Belgrade). The preparations were allowed to equilibrate for 15 min under an initial tension of 0.5 g following which acetylcholine (1–100 μM) in the absence and presence of menthol (0.1 μM) was applied to the preparation. The responses for each concentration were expressed in grams (g).

5.4.4 Drug Applications

Acetylcholine chloride, L-menthol, (-)-menthone, eugenol, (S)-(-)-*β*-citronellol, (+)-limonene oxide, carvacrol, ((R)-(-))-carvone, geraniol, menthyl acetate, phenethyl propionate, *trans*-cinnamaldehyde and BAPTA-AM were purchased from Sigma-Aldrich (St Louis, MO, USA). (+)-Pulegone was purchased from Kodak (Rochester, NY, USA) and geraniol from Berje (Carteret, NJ, USA). Acetylcholine was dissolved in recording solution while monoterpenoid compounds (Figure 4.0) were dissolved in DMSO such that the final working concentration did not exceed 0.1%.

Oocytes were perfused with drugs by an 8-channel system (VC-8 valve controller) (Warner Instruments, USA) at a flow rate of ~6 mL/min. For the agonist experiments, all the essential oils were used at a concentration of 100 μM. All the responses were normalized to the control (100 μM acetylcholine) response. Each drug was applied for 10s and the wash off time between each drug was 2 minutes. The application sequence of

agonists for determining the potency series was random and not predetermined. The concentration-response relationship for acetylcholine was generated by applying increasing concentrations of acetylcholine (0.1-300 μM) for 10 s. For positive allosteric modulator experiments, we tested the effects of menthol (0.1 and 10 μM) on acetylcholine and levamisole concentration-response relationships. Control concentration-responses were obtained by applying ascending concentrations of the agonist for 10 s. Concentration-response relationships in the presence of 0.1 μM and 10 μM menthol were generated by first applying a control 100 μM acetylcholine for 10 s, followed by a two min application of menthol, and 10 s applications of increasing concentrations of the agonist in the continued presence of menthol. Between each drug application a 2 min wash off time was allowed, and the responses were normalized to the control 100 μM acetylcholine current.

In order to characterize the antagonist properties of the phytocompounds, a control concentration of 100 μM ACh was first applied for 30 s followed by a 2 min wash with recording solution. Thereafter, 100 μM acetylcholine was applied for 10 s, immediately followed by 10 s application of the antagonist (100 μM) in the continued presence of 100 μM ACh and then a final 10 s application of 100 μM ACh. At least 2 min drug wash off interval was allowed between applications in order to minimize desensitization. Note that, the potency for each antagonist is likely to be slightly underestimated due to the short time of drug application with this approach (Abongwa, Buxton, et al. 2016). Acetylcholine concentration-response relationships in the presence of 100 μM limonene oxide and 100 μM carvacrol were generated by first applying a

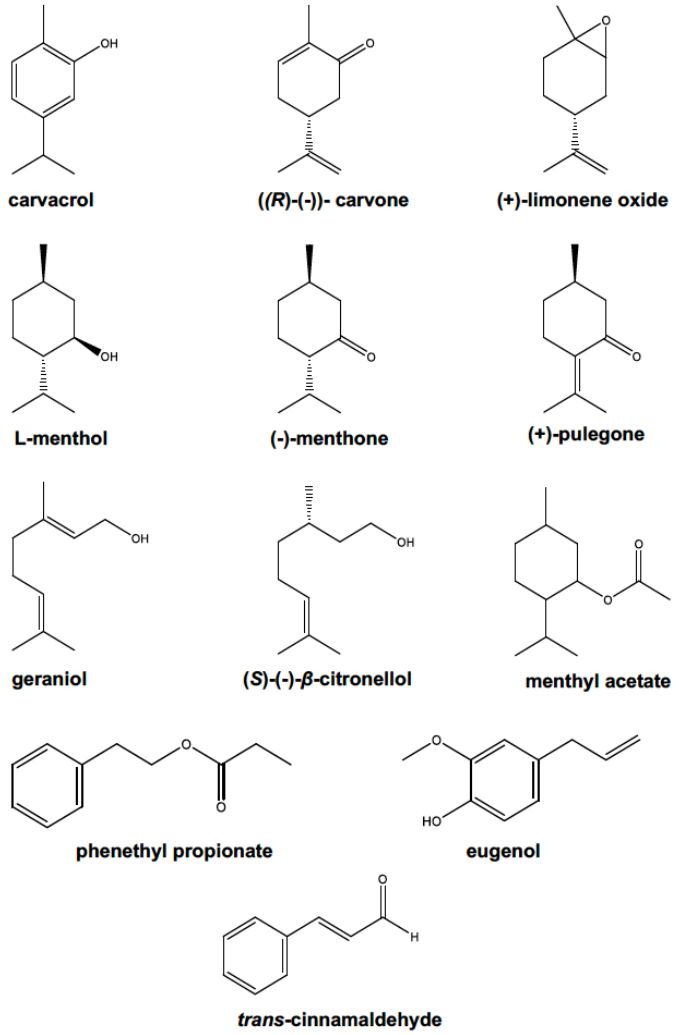

Figure 5.0 Chemical structures of monoterpenoid compounds used in the present study.

control 100 μM acetylcholine for 10 s, followed by a 2-min application of the antagonist, and 10 s applications of 3, 10, 30, 100 and 300 μM acetylcholine in the continued presence of antagonist. The responses were normalized to the control 100 μM acetylcholine current.

5.4.5 Data and statistical analysis

Data acquired from electrophysiological recordings were analyzed with Clampfit 10.3 (Molecular Devices, Sunnyvale, CA, USA) and GraphPad Prism 7.0 (Graphpad Software Inc., La Jolla, CA, USA). The peak current responses in oocytes to applied

drugs were normalized to the 100 μM ACh control current (unless otherwise indicated) and expressed as mean ± SEM. All completed drug application sequences on the oocytes were used for analysis without exclusion. If the recording became unstable, indicated by a change in the baseline holding current, all of that recording was rejected for analysis. The mean % inhibition produced by monoterpenoid compounds on currents elicited by 100 μM acetylcholine were calculated as previously described (Zheng et al. 2016). For *A. suum* contraction experiments, mean contraction responses in grams (g) to each concentration of acetylcholine in the absence and presence of menthol was determined. The sigmoidal concentration-responses for the oocyte experiments and isometric contraction studies were described using the following equation:

$$\% \text{ response} = 1/1+[EC_{50}/Xa]^{nH}$$

where EC_{50} is the concentration of agonist (Xa) producing 50% of the maximum response and nH is the Hill coefficient (slope). Differences in pEC_{50} and I_{max} were assessed using extra sum of squares *F*-test. Two-way ANOVA and paired *t*-tests were used to test differences between control contractions and test contractions on the same muscle flap. The difference was considered significant if $P<0.05$.

5.5 Results

5.5.1 Pharmacology of monoterpenoids on *Ode* (29-38-63-8) receptor

5.5.1.1. Agonist pharmacology

The agonist properties of 12 monoterpenoid compounds were investigated on the levamisole sensitive *Ode-unc-29:Ode-unc-38:Ode-unc-63:Ode-acr-8* nAChR (Figure 5.1). 11 of the 12 monoterpenoids we tested showed no significant agonistic effect

(<1.0% of control acetylcholine current) on the expressed levamisole sensitive channel. Menthol was the only compound tested that produced an agonist effect, showing only ≈ 6.5% of the control acetylcholine currents.

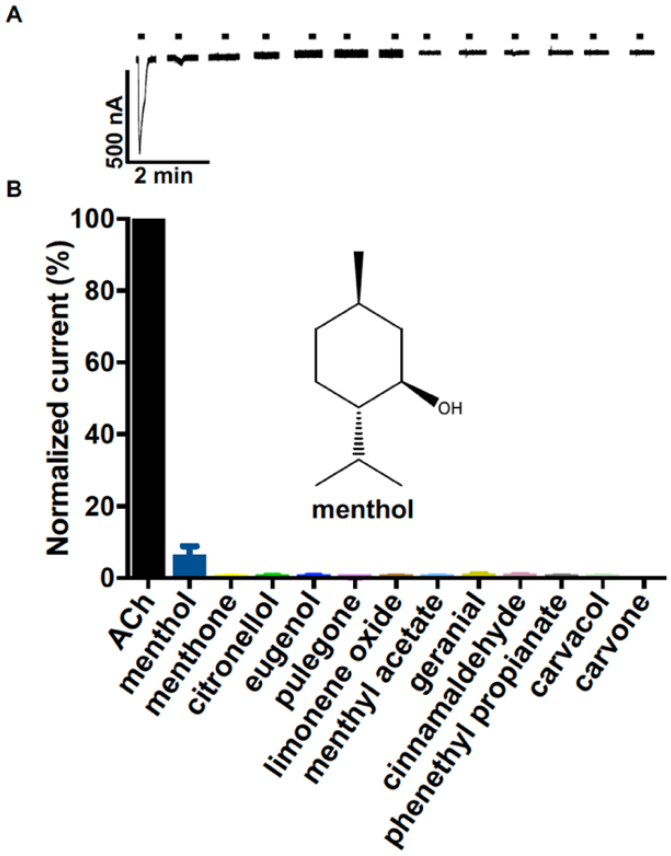

Figure 5.1 Agonist effects of monoterpenoid compounds on levamisole sensitive *O. dentatum* nAChR. A. Sample traces for the agonist experiment. B. Bar chart (expressed as mean ± SEM%) showing agonistic effects of the monoterpenoid compounds: menthol (6.5 ± 2.4%, n = 6), geraniol (0.9 ± 0.2%, n = 6), cinnamaldehyde (0.6 ± 0.2%, n = 6), citronellol (0.4 ± 0.2%, n = 6), menthyl acetate (0.3 ± 0.1%, n = 6), eugenol (0.3 ± 0.2%, n = 6), phenethyl propionate (0.3 ± 0.1%, n = 6), limonene oxide (0.3 ± 0.2%, n = 6), carvacrol (0.2 ± 0.2%, n = 6), menthone (0.2 ± 0.1%, n = 6), pulegone (0.1 ± 0.1%, n = 6) and carvone (0.0 ± 0.0%, n = 6).

5.5.1.2. Antagonist pharmacology

We tested potential antagonistic effects of the same 12 monoterpenoids on the levamisole sensitive receptor (Figure 5.2). Among all the phytocompounds tested limonene oxide, citronellol, carvone, carvacrol, pulegone and eugenol reduced the

acetylcholine response. Limonene oxide was the most potent inhibitor (mean inhibition% = 36.0 ± 3.2%). The rank order potency of inhibition was: limonene oxide > citronellol > carvone > carvacrol = pulegone = eugenol (Figure 5.2A). Interestingly, monoterpenoids including menthol, menthone, menthyl acetate and geraniol increased the amplitude of currents produced by acetylcholine instead of inhibiting them (data not shown). Menthol produced the most potent positive allosteric modulation and increased the response by 3.1 ± 0.7% (mean ± SEM) with a maximum potentiation of 6.3%.

5.5.1.3. Antagonistic effects of limonene oxide and carvacrol on acetylcholine concentration-response relationship

The concentration-response relationship for acetylcholine was examined by applying increasing concentrations of acetylcholine 0.3–300 μM to oocytes expressing the levamisole sensitive nAChR (Figure 5.2 B&C). The sigmoidal concentration-response fit gave pEC_{50} = 5.3 ± 0.0 (EC_{50} = 5.3 μM), a hillslope (n_H) of 2.0 ± 0.2 and I_{max} value of 104.6 ± 1.6%. We investigated the effects of limonene oxide (100 μM) and carvacrol (100 μM) on the acetylcholine concentration-response relationship. I_{max} for acetylcholine in the presence of limonene oxide and carvacrol were 62.8 ± 7.0% and 74.1 ± 3.2%, respectively. Both the monoterpenoid compounds produced a statistically significant reduction in the maximum response (P<0.001 for limonene oxide; P<0.0001 for carvacrol; Figure 5.2 B&C). Neither carvacrol, pEC_{50} = 5.7 ± 0.1 (EC_{50} = 2.0 μM) nor limonene oxide, pEC_{50} = 5.4 ± 0.2 (EC_{50} = 4.0 μM) increased the EC_{50} values c.f. control. This indicates limonene oxide and carvacrol both produced non-competitive inhibition and are not binding at the agonist-binding site to produce their effect.

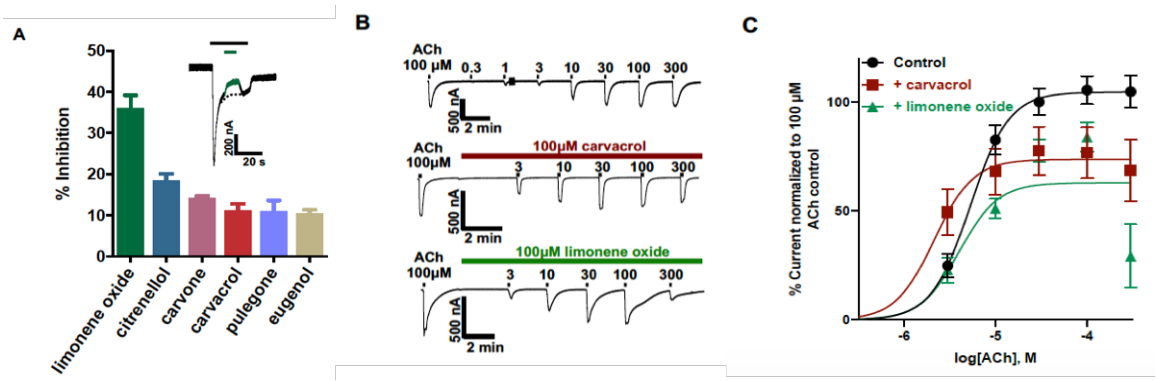

Figure 5.2: Effect of monoterpenoid compounds as antagonists on levamisole sensitive *O. dentatum* receptor acetylcholine mediated responses. A. Bar graph showing the rank order potency of monoterpenoid compounds as antagonists. Results were expressed as % mean inhibition ± SEM of currents elicited by 100 µM ACh: limonene oxide (36.0 ± 3.2%, n = 6) > citronellol (18.0 ± 1.6%, n = 6) > carvone (14.0 ± 0.6%, n = 6) > carvacrol (11.0 ± 1.6%, n = 6) = pulegone (11.0 ± 2.6%, n = 6) = eugenol (11.0 ± 1.0 %, n = 6). Inset: image showing predicted acetylcholine response in the absence of limonene oxide (dotted line) and inhibition of acetylcholine mediated response in the presence of limonene oxide (highlighted in green). B. Acetylcholine current responses, recorded from the *Xenopus* oocyte expressing the levamisole sensitive channel, alone and in the presence of 100µM carvacrol (n=6) & 100 µM limonene oxide (n = 5). C. Concentration-response plots for acetylcholine alone (n = 6, black) and acetylcholine in the presence of 100 µM carvacrol (n = 6, maroon) & 100 µM limonene oxide (n = 5, green). pEC_{50} (mean ± SEM), EC_{50} (mean, µM), Hill slope (n_H) (mean ± SEM) and I_{max} (mean ± SEM%) values were respectively: 5.3 ± 0.0, 5.3 µM, 2.0 ± 0.2 and 104.6 ± 1.6 for acetylcholine alone; 5.4 ± 0.2, 4.0 µM, 2.2 ± 1.7 and 62.8 ± 7.0 in the presence of limonene oxide; 5.7 ± 0.1, 2.0 µM, 1.7 ± 1.1 and 74.1 ± 3.2 in the presence of carvacrol. Bottom was constrained to zero for curve fitting.

5.5.1.4. Menthol as positive allosteric modulator

Menthol was a weak agonist on the expressed levamisole sensitive receptor. However, it appeared to potentiate acetylcholine responses in our antagonist experiments (data not shown). To investigate positive allosteric modulation further, we analyzed the effect of menthol on acetylcholine and levamisole concentration-response relationships (Figure 5.3 and Figure 5.4). We observed a left shift in the sigmoidal concentration-response curves for both levamisole and acetylcholine in the presence of menthol. Sensitivity of the receptor was increased significantly for acetylcholine, pEC_{50} = 5.3 ± 0.0 (EC_{50} = 5.0 µM); in the presence of 0.1 µM menthol, pEC_{50} = 6.4 ± 0.1 (EC_{50} = 0.4 µM) (EC_{50} = 5.0 µM);

in the presence of 0.1 µM menthol, pEC_{50} = 6.4 ± 0.1 (EC_{50} = 0.4 µM) and with 10 µM menthol, pEC_{50} = 6.5 ± 0.1 (EC_{50} = 0.3 µM).

For levamisole alone, pEC_{50} and EC_{50} values were 6.3 ± 0.1 and 0.5 µM respectively. In the presence of 0.1 µM menthol pEC_{50} and EC_{50} values were 6.5 ± 0.1 and 0.3 µM respectively. In the presence of 10 µM menthol the pEC_{50} and EC_{50} values were 6.8 ± 0.2 and 0.2 µM. Only 10 µM menthol produced a significant left shift in the levamisole concentration-response curve (P<0.05; Figure 5.4C). The positive allosteric modulation produced by menthol was more pronounced with acetylcholine as an agonist in comparison to levamisole. In conclusion, menthol not only acts as an agonist on the levamisole sensitive receptor but also displays significant PAM activity on this nAChR.

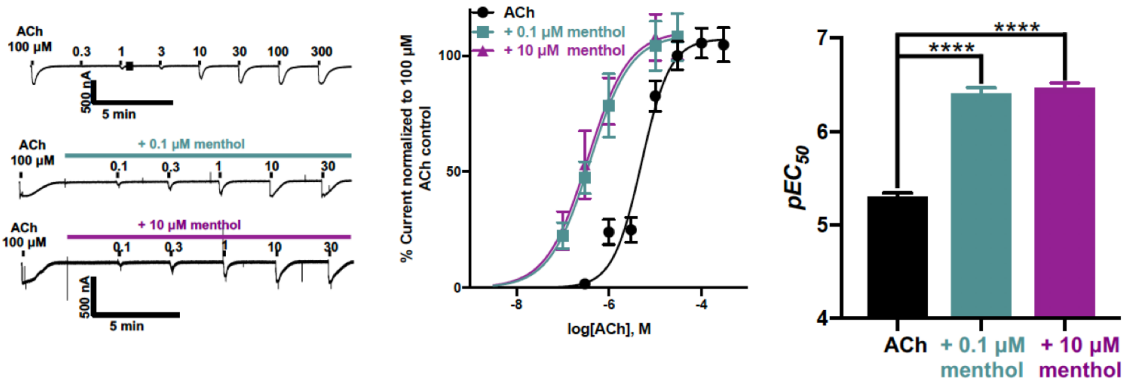

Figure 5.3: Effects of menthol as a PAM on levamisole sensitive *O. dentatum* nAChR acetylcholine responses. A. Sample traces for two-electrode voltage-clamp recording showing inward currents in response to ascending concentrations of acetylcholine alone, acetylcholine in the presence of 0.1 µM and 10 µM menthol. B. Concentration-response relationships for acetylcholine alone (n = 7, black), acetylcholine in the presence of 0.1 µM (n = 6, blue) and 10 µM menthol (n=7, purple). pEC_{50} (mean ± SEM), EC_{50} (mean, µM), Hill slope (n_H) (mean ± SEM) and I_{max} (mean ± SEM%) values were respectively: 5.3 ± 0.0, 5.0 µM, 1.4 ± 0.1 and 107.5 ± 2.6 for acetylcholine alone; 6.4 ± 0.1, 0.4 µM, 1.0 ± 0.1 and 109.0 ± 3.6 in the presence of 0.1 µM menthol; 6.5 ± 0.1, 0.3 µM, 1.0 ± 0.1 and 110.5 ± 3.7 in the presence of 10 µM menthol. Bottom was constrained to zero for curve fitting. C. Bar chart showing comparison of pEC_{50} (expressed as mean ± SEM) for acetylcholine in the presence and absence of menthol. ****P<0.0001; significantly different as indicated; extra sum of squares F-test.

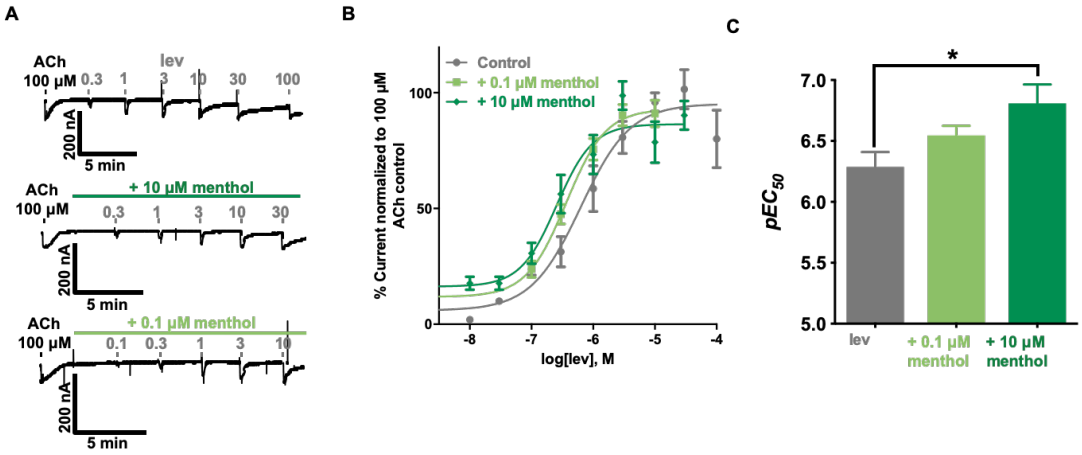

Figure 5.4: Effects of menthol on levamisole sensitive nAChR levamisole responses. A. Representative current traces for two-electrode voltage-clamp recording showing inward currents in response to ascending application of levamisole alone, levamisole in the presence of 0.1 µM and 10 µM menthol. B. Concentration-response relationships for levamisole alone (n = 7, steel gray) and in the presence of 0.1 µM (n = 6, light green) and 10 µM menthol (n=7, dark green). pEC_{50} (mean ± SEM), EC_{50} (mean, µM), Hill slope (n_H) (mean ± SEM) and I_{max} (mean ± SEM%) values were respectively: 6.3 ± 0.1, 0.5 µM, 1.0 ± 0.2 and 96.3 ± 5.2 for levamisole alone; 6.5 ± 0.1, 0.3 µM, 1.1 ± 0.2 and 94.6 ± 5.0 in the presence of 0.1 µM menthol; 6.8 ± 0.2, 0.2 µM, 1.0 ± 0.2 and 89.2 ± 6.0 in the presence of 10 µM menthol. Bottom was constrained to zero for curve fitting. C. Bar chart summarizing the results showing comparison of pEC_{50} (expressed as mean ± SEM) for levamisole in the presence and absence of menthol. *P<0.05; significantly different as indicated; extra sum of squares F-test.

5.5.2 Effects of menthol and carvacrol on *A. suum* ACR-16 nAChR

Figure 5.5 shows the concentration-response relationship for acetylcholine on the expressed *Asu*-ACR-16 nicotine sensitive nAChR. The sigmoidal concentration-response fit gave pEC_{50} = 5.0 ± 0.1 (EC_{50} = 11.1 µM), I_{max} = 109.1 ± 6.6% and a hillslope (n_H) of 1.6 ± 0.3. We investigated the effects of menthol (10 µM) and carvacrol (10 µM and 100 µM) on the acetylcholine response. There was no significant change in the sensitivity or efficacy of acetylcholine on the N-type receptor in the presence of menthol (pEC_{50} = 5.0 ± 0.1 and I_{max} =101.6 ± 7.5%). The concentration-response fits (Figure 5.5C) show that 100 µM carvacrol decreased the maximum response while 10 µM carvacrol failed to exhibit any significant antagonistic properties. In the presence of 10 µM carvacrol $pEC_{50,}$

EC_{50} and I_{max} values were 5.1 ± 0.1, 8.1 µM and 94.6 ± 5.9% respectively. Carvacrol produced a significant reduction in I_{max} (55.8 ± 4.4%; P<0.05) when applied at 100 µM concentration but did not produce a significant right shift in the pEC_{50} (5.1 ± 0.1). This indicates carvacrol at 100 µM produced non-competitive inhibition.

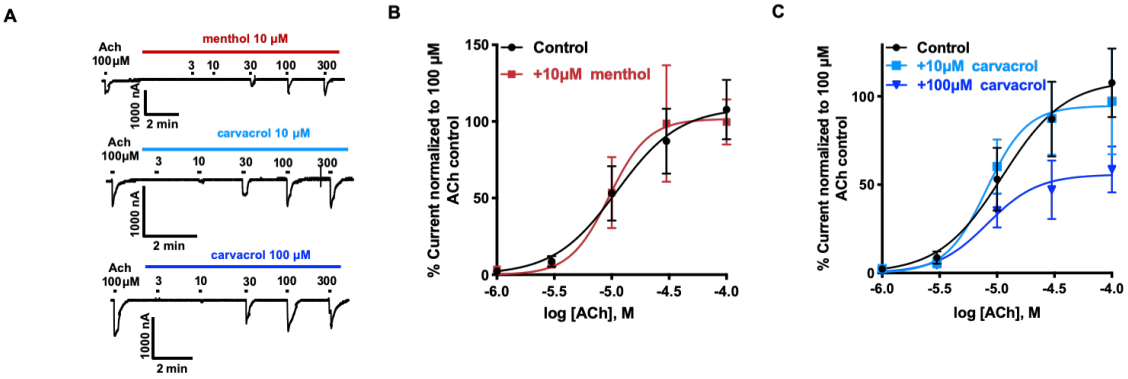

Figure 5.5 Effects of menthol and carvacrol on nicotine sensitive *A. suum* ACR-16 nAChR acetylcholine responses. A. Representative current traces for two-electrode voltage-clamp recording showing inward currents in response to increasing acetylcholine concentrations in the presence of menthol (10 µM) and carvacrol (10 µM and 100 µM) B. Concentration-response plot for acetylcholine alone (n = 7, black) and acetylcholine in the presence of menthol (n = 6, red). pEC_{50} (mean ± SEM) and EC_{50} (mean, µM) values were respectively: 5.0 ± 0.1 and 11.1 µM for acetylcholine alone, 5.0 ± 0.1 and 9.5 µM in the presence of 10 µM menthol. Hill slope (n_H) (mean ± SEM) values were: 1.6 ± 0.3 for acetylcholine alone; 2.5 ± 1.2 in the presence of 10 µM menthol. I_{max} (mean ± SEM%) values were: 109.1 ± 6.6 for acetylcholine alone; 101.6 ± 7.5 in the presence of 10 µM menthol. Bottom was constrained to zero for curve fitting. C. Concentration-response plots for acetylcholine alone (n = 7, black) and in the presence of carvacrol (n=6, blue). pEC_{50} (mean ± SEM), EC_{50} (mean, µM), Hill slope (n_H) (mean ± SEM) and I_{max} (mean ± SEM%) values were respectively: 5.1 ± 0.1, 8.1 µM, 2.5 ± 0.8 and 94.6 ± 5.9 in the presence of 10 µM carvacrol; 5.1 ± 0.1, 8.2 µM, 2.0 ± 0.7 and 55.8 ± 4.4 in the presence of 100 µM carvacrol. Bottom was constrained to zero for curve fitting.

5.5.3 Effect of menthol on *A. suum* muscle

We examined the effect of menthol on the *A. suum* muscle flap contractions induced by increasing concentrations of acetylcholine. Figure 5.6A shows the representative trace for acetylcholine, in the absence and presence of 0.1 µM menthol in the experimental bath. Acetylcholine produced concentration-dependent isometric

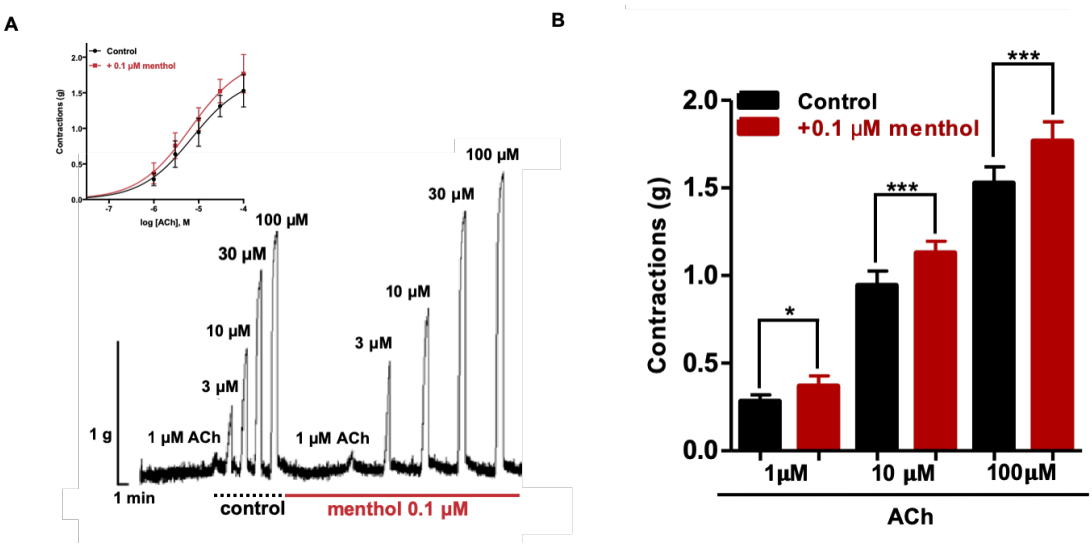

Figure 5.6: A. Sample trace for the *A. suum* muscle flap contraction experiments. Inset: concentration-response plots for acetylcholine alone (n = 6, black) and in the presence of 0.1 µM menthol (n = 6, red). pEC_{50} (mean ± SEM) and EC_{50} (mean, µM) values were respectively: 5.2 ± 0.2 and 7.0 µM for acetylcholine alone; 5.2 ± 0.2 and 6.5 µM in the presence of 0.1 µM menthol. R_{max} (mean ± SEM, g) values were: 1.7 ± 0.2 g for acetylcholine alone and 2.0 ± 0.2 g in the presence of 0.1 µM menthol. Hill slope (n_H) (mean ± SEM) values were: 0.8 ± 0.2 for acetylcholine alone and 0.8 ± 0.1 in the presence of 0.1 µM menthol. Bottom was constrained to zero for curve fitting. B. Bar chart summarizing the results showing significant potentiation in amplitude of acetylcholine mediated contractions in the presence of 0.1 µM menthol. *P<0.05, ***P<0.001; significantly different as indicated; paired *t*-tests.

contractions with a pEC_{50} of 5.2 ± 0.2 (EC_{50} = 7.0 µM) and R_{max} = 1.7 ± 0.2 g. We did not observe a significant left shift in pEC_{50} (5.2 ± 0.2) or change in R_{max} (2.0 ± 0.2 g) in the presence of menthol. As expected, based on two-way ANOVA, acetylcholine concentration had a significant effect on muscle contraction (P<0.0001). Importantly the presence of menthol also had a significant effect on muscle contraction (P<0.0001). Further analysis revealed a significant potentiation of contractions at each concentration of acetylcholine in the presence of menthol (paired *t*-tests, P<0.05, Figure 5.6B for examples). This modulatory effect of menthol is similar to our observed effects on the levamisole sensitive receptor.

5.6 Discussion

Monoterpenoids are a group of plant secondary metabolites that have been shown to modulate the function of nicotinic acetylcholine receptors of mammals and insects (Lozon et al. 2016; Tong et al. 2013; Park et al. 2003; Park et al. 2001). We were interested in finding possible agonists, antagonists or modulators for these cys-loop receptors of parasitic nematodes. In this study, we provide evidence of significant modulatory effects of several monoterpenoid compounds on the levamisole sensitive *O. dentatum* receptor and nicotine sensitive *A. suum* nAChR. We chose to use the levamisole sensitive nAChR from *O. dentatum* due to unreliable expression of the levamisole sensitive nAChR from *A. suum* in our hands.

The majority of the phytocompounds tested failed to exhibit agonist properties on the nAChRs but we were able to identify inhibitors of the levamisole sensitive nematode receptor with a rank order: limonene oxide > citronellol > carvone > carvacrol = pulegone = eugenol.

Limonene oxide and carvacrol produced significant non-competitive inhibition suggesting neither compound acts at the ligand binding sites. Limonene oxide is a monoterpene found in essential oils of various citrus fruits and is an odorous component of some types of mushrooms (Breheret et al. 1997; Lemes et al. 2018; Lota et al. 2002; Vieira et al. 2018; Rapior et al. 1997); it has been reported to stimulate TRPA1 cation channels (Kaimoto et al. 2016). There are limited data available on the effects of limonene oxide on nAChRs and this is the first example of an antagonistic effect of the compound on a levamisole sensitive nematode nAChR. According to a study done by Kim et al. (2013) limonene oxide was reported to be a safe phytochemical in mammals

and thus could be used in combination with other nicotinic inhibitors for anti-nematodal therapy. Carvacrol, found in many plants including thyme, oregano and Alaska yellow cedar (Bouchra et al. 2003; De Vincenzi et al. 2004), acted as a non-competitive inhibitor on both levamisole sensitive and nicotine sensitive nematode receptors. Various studies have shown alpha-7 homomeric nAChRs, GABA (gamma amino-butyric acid) and tyramine receptors as target sites for carvacrol (Lozon et al. 2016; Tong et al. 2013; Tong and Coats 2012). In addition, carvacrol was shown to produce significant inhibitory effects on acetylcholine induced muscle contractions in *A. suum* (Trailović et al. 2015). This polypharmacological effect of carvacrol might make it efficacious as an anthelmintic phytotherapeutic, although it may be best considered as an adjunct to other anti-nematodal compounds to increase efficacy due to toxicity considerations when used alone at high concentrations (Bimczok et al. 2008; Roselli et al. 2007; Stammati et al. 1999).

The most interesting finding was the positive allosteric modulatory activity of menthol on the levamisole sensitive nematode nAChR. Menthol is a monocyclic terpene alcohol extracted from peppermint plants, *Mentha* spp. and regulates somatosensory sensations such as warmth, pain and irritation (Cliff and Green 1994; Eccles 1994; Farco and Grundmann 2013). Menthol showed very mild agonist activity but produced statistically significant potentiation of acetylcholine induced inward currents at concentrations as low as 0.1 μM and of levamisole induced currents at 10 μM on the levamisole sensitive cation channel. PAM compounds exhibit potentiation of the agonist-activation effects by binding to a site distinct from the ligand binding sites. This property is highly desired in drug discovery and development as PAMs can achieve subtype

selectivity more easily in comparison to compounds binding to orthosteric sites (Chatzidaki and Millar 2015; Williams, Wang, and Papke 2011) as all the nAChRs have highly conserved acetylcholine binding sites. In comparison, the conservation for amino acid composition for other sites is less which makes PAMs preferable in terms of selectivity and reduced potential off target effects (Chatzidaki and Millar 2015; Williams, Wang, and Papke 2011). Menthol modulates the pharmacological properties and expression of vertebrate nicotinic acetylcholine receptors; it inhibits the effect of nicotine on α7-nACh receptors in neural cells in a non-competitive manner (Ashoor et al. 2013; Hans, Wilhelm, and Swandulla 2012; Ton et al. 2015). However, it is worth noting that these negative allosteric modulatory and inhibitory effects of menthol on α7-nACh receptors were produced by much higher concentrations (30-300 μM) in comparison to our study. In addition, menthol at 10 μM had no significant effect on the nematode nicotine sensitive nAChR (the closest ortholog to vertebrate α7-nAChRs in the nematodes). This makes menthol an interesting candidate that can potentiate the effects of cholinergic agonist anthelmintics acting on levamisole sensitive nAChRs. Importantly, 0.1 μM menthol produced significant potentiation of contractions produced by acetylcholine on *A. suum* muscle flaps. The presence of positive allosteric modulation in the somatic muscle flap preparation can be attributed to the presence of levamisole receptors and is important *in vivo* confirmation of our findings from the *in vitro* heterologous expression studies.

5.7 Conclusion

Natural products have formed the basis of sophisticated traditional therapies and they continue to play essential roles in modern healthcare. Phytocompounds, with their complex chemistry and structural diversity, offer the potential for discovery and development of new pharmaceuticals. Botanical anthelmintics may reduce the need for chemical drug treatment and alleviate the pressure on the limited group of anthelmintics available. Carvacrol produced significant antagonism on the acetylcholine induced inward currents on both levamisole sensitive and nicotine sensitive nematode ligand-gated ion channels. This illustrates a multifaceted mode of action with involvement of multiple target subtypes that can help ameliorate the issue of drug resistance. It may be possible to combine these compounds with the nAChR antagonist derquantel or perhaps with a macrocyclic lactone as with Startect® (derquantel + abamectin).

Our results provide promising evidence of positive allosteric modulation by menthol which could be used in combination therapy with cholinomimetic drugs like pyrantel or levamisole to increase efficacy. For future investigations menthol could be used as the starting point in structure activity relationship studies to find new potent PAM compounds suitable for stand-alone use as anthelmintics. In summary, the use of plant derived compounds, either alone or in combination, could facilitate sustainable control of parasitic nematodes. The results of the present study are encouraging and suggest monoterpenoids can be exploited as components of new anthelmintic formulations.

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5.9 References

Abongwa, M., K. E. Baber, R. J. Martin, and A. P. Robertson. 2016. 'The cholinomimetic morantel as an open channel blocker of the Ascaris suum ACR-16 nAChR', *Invert Neurosci*, 16: 10.

Abongwa, M., S. K. Buxton, E. Courtot, C. L. Charvet, C. Neveu, C. J. McCoy, S. Verma, A. P. Robertson, and R. J. Martin. 2016. 'Pharmacological profile of Ascaris suum ACR-16, a new homomeric nicotinic acetylcholine receptor widely distributed in Ascaris tissues', *Br J Pharmacol*, 173: 2463-77.

Abongwa, M., R. J. Martin, and A. P. Robertson. 2017. 'A Brief Review on the Mode of Action of Antinematodal Drugs', *Acta Vet (Beogr)*, 67: 137-52.

Albonico, M., Q. Bickle, M. Ramsan, A. Montresor, L. Savioli, and M. Taylor. 2003. 'Efficacy of mebendazole and levamisole alone or in combination against intestinal nematode infections after repeated targeted mebendazole treatment in Zanzibar', *Bull World Health Organ*, 81: 343-52.

Alum, A., J. R. Rubino, and M. K. Ijaz. 2010. 'The global war against intestinal parasites--should we use a holistic approach?', *Int J Infect Dis*, 14: e732-8.

Anquez-Traxler, Christelle. 2011. 'The Legal and Regulatory Framework of Herbal Medicinal Products in the European Union: A Focus on the Traditional Herbal Medicines Category', *Drug Information Journal*, 45: 15-23.

Ashoor, A., J. C. Nordman, D. Veltri, K. H. Yang, L. Al Kury, Y. Shuba, M. Mahgoub, F. C. Howarth, B. Sadek, A. Shehu, N. Kabbani, and M. Oz. 2013. 'Menthol binding and inhibition of alpha7-nicotinic acetylcholine receptors', *PLoS One*, 8: e67674.

Balick, M. J., and P.A. Cox. 1996. *Plants, people, and culture: the science of ethnobotany/ Michael J. Balick, Paul Alan Cox (Scientific American Library series no. 60)*. (New York : Scientific American Library).

Balunas, M. J., and A. D. Kinghorn. 2005. 'Drug discovery from medicinal plants', *Life Sci*, 78: 431-41.

Bethony, J., S. Brooker, M. Albonico, S. M. Geiger, A. Loukas, D. Diemert, and P. J. Hotez. 2006. 'Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm', *Lancet*, 367: 1521-32.

Bimczok, D., H. Rau, E. Sewekow, P. Janczyk, W. B. Souffrant, and H. J. Rothkotter. 2008. 'Influence of carvacrol on proliferation and survival of porcine lymphocytes and intestinal epithelial cells in vitro', *Toxicol In Vitro*, 22: 652-8.

Bouchra, C., M. Achouri, L. M. Idrissi Hassani, and M. Hmamouchi. 2003. 'Chemical composition and antifungal activity of essential oils of seven Moroccan Labiatae against Botrytis cinerea Pers: Fr', *J Ethnopharmacol*, 89: 165-9.

Breheret, Sophie, Thierry Talou, Sylvie Rapior, and Jean-Marie Bessière. 1997. 'Monoterpenes in the Aromas of Fresh Wild Mushrooms (Basidiomycetes)', *Journal of Agricultural and Food Chemistry*, 45: 831-36.

Brooker, S. 2010. 'Estimating the global distribution and disease burden of intestinal nematode infections: adding up the numbers--a review', *Int J Parasitol*, 40: 1137-44.

Brooker, S., A. C. Clements, and D. A. Bundy. 2006. 'Global epidemiology, ecology and control of soil-transmitted helminth infections', *Adv Parasitol*, 62: 221-61.

Butler, M. S. 2004. 'The role of natural product chemistry in drug discovery', *J Nat Prod*, 67: 2141-53.

Buxton, S. K., C. L. Charvet, C. Neveu, J. Cabaret, J. Cortet, N. Peineau, M. Abongwa, E. Courtot, A. P. Robertson, and R. J. Martin. 2014. 'Investigation of acetylcholine receptor diversity in a nematode parasite leads to characterization of tribendimidine- and derquantel-sensitive nAChRs', *PLoS Pathog*, 10: e1003870.

Calapai, G. 2008. 'European legislation on herbal medicines: a look into the future', *Drug Saf*, 31: 428-31.

Camurca-Vasconcelos, A. L., C. M. Bevilaqua, S. M. Morais, M. V. Maciel, C. T. Costa, I. T. Macedo, L. M. Oliveira, R. R. Braga, R. A. Silva, and L. S. Vieira. 2007.

'Anthelmintic activity of Croton zehntneri and Lippia sidoides essential oils', *Vet Parasitol*, 148: 288-94.

Cetin, H., J. E. Cilek, L. Aydin, and A. Yanikoglu. 2009. 'Acaricidal effects of the essential oil of Origanum minutiflorum (Lamiaceae) against Rhipicephalus turanicus (Acari: Ixodidae)', *Vet Parasitol*, 160: 359-61.

Chatzidaki, A., and N. S. Millar. 2015. 'Allosteric modulation of nicotinic acetylcholine receptors', *Biochem Pharmacol*, 97: 408-17.

Cliff, M. A., and B. G. Green. 1994. 'Sensory irritation and coolness produced by menthol: evidence for selective desensitization of irritation', *Physiol Behav*, 56: 1021-9.

Coats, J. R. 1994. 'Risks from natural versus synthetic insecticides', *Annu Rev Entomol*, 39: 489-515.

Committee on the Use of Complementary, and Alternative Medicine by the American Public, Board on Health Promotion, and Disease Prevention, Institute of Medicine. 2005. *Complementary and Alternative Medicine in the United States* (The National Academies Press: Washington, DC).

Coskun, S., O. Girisgin, M. Kurkcuoglu, H. Malyer, A. O. Girisgin, N. Kirimer, and K. H. Baser. 2008. 'Acaricidal efficacy of Origanum onites L. essential oil against Rhipicephalus turanicus (Ixodidae)', *Parasitol Res*, 103: 259-61.

De Clercq, D., M. Sacko, J. Behnke, F. Gilbert, P. Dorny, and J. Vercruysse. 1997. 'Failure of mebendazole in treatment of human hookworm infections in the southern region of Mali', *Am J Trop Med Hyg*, 57: 25-30.

De Vincenzi, M., A. Stammati, A. De Vincenzi, and M. Silano. 2004. 'Constituents of aromatic plants: carvacrol', *Fitoterapia*, 75: 801-4.

Dias, D. A., S. Urban, and U. Roessner. 2012. 'A historical overview of natural products in drug discovery', *Metabolites*, 2: 303-36.

Eccles, R. 1994. 'Menthol and related cooling compounds', *J Pharm Pharmacol*, 46: 618-30.

Enan, E. E. 2005. 'Molecular and pharmacological analysis of an octopamine receptor from American cockroach and fruit fly in response to plant essential oils', *Arch Insect Biochem Physiol*, 59: 161-71.

Farco, J. A., and O. Grundmann. 2013. 'Menthol--pharmacology of an important naturally medicinal "cool"', *Mini Rev Med Chem*, 13: 124-31.

Ferrell, J. A. 1914. *The Rural School and Hookworm Disease* (Government Printing Office, : Washington D.C).

Fincham, J. E., M. B. Markus, and V. J. Adams. 2003. 'Could control of soil-transmitted helminthic infection influence the HIV/AIDS pandemic', *Acta Trop*, 86: 315-33.

Flohr, C., L. N. Tuyen, S. Lewis, T. T. Minh, J. Campbell, J. Britton, H. Williams, T. T. Hien, J. Farrar, and R. J. Quinnell. 2007. 'Low efficacy of mebendazole against hookworm in Vietnam: two randomized controlled trials', *Am J Trop Med Hyg*, 76: 732-6.

Hans, M., M. Wilhelm, and D. Swandulla. 2012. 'Menthol suppresses nicotinic acetylcholine receptor functioning in sensory neurons via allosteric modulation', *Chem Senses*, 37: 463-9.

Harvey, A. L., R. Edrada-Ebel, and R. J. Quinn. 2015. 'The re-emergence of natural products for drug discovery in the genomics era', *Nat Rev Drug Discov*, 14: 111-29.

Heinrich, M., and H. Lee Teoh. 2004. 'Galanthamine from snowdrop--the development of a modern drug against Alzheimer's disease from local Caucasian knowledge', *J Ethnopharmacol*, 92: 147-62.

Hotez, P. J., P. J. Brindley, J. M. Bethony, C. H. King, E. J. Pearce, and J. Jacobson. 2008. 'Helminth infections: the great neglected tropical diseases', *J Clin Invest*, 118: 1311-21.

Hu, D., and J. Coats. 2008. 'Evaluation of the environmental fate of thymol and phenethyl propionate in the laboratory', *Pest Manag Sci*, 64: 775-9.

Jaradat, N., L. Adwan, S. K'Aibni, N. Shraim, and A. N. Zaid. 2016. 'Chemical composition, anthelmintic, antibacterial and antioxidant effects of Thymus bovei essential oil', *BMC Complement Altern Med*, 16: 418.

Kaimoto, T., Y. Hatakeyama, K. Takahashi, T. Imagawa, M. Tominaga, and T. Ohta. 2016. 'Involvement of transient receptor potential A1 channel in algesic and analgesic actions of the organic compound limonene', *Eur J Pain*, 20: 1155-65.

Kaplan, R. M., B. E. Storey, A. N. Vidyashankar, B. W. Bissinger, S. M. Mitchell, S. B. Howell, M. E. Mason, M. D. Lee, A. A. Pedroso, A. Akashe, and D. J. Skrypec. 2014. 'Antiparasitic efficacy of a novel plant-based functional food using an Ascaris suum model in pigs', *Acta Tropica*, 139: 15-22.

Katiki, L. M., A. C. Chagas, H. R. Bizzo, J. F. Ferreira, and A. F. Amarante. 2011. 'Anthelmintic activity of Cymbopogon martinii, Cymbopogon schoenanthus and Mentha piperita essential oils evaluated in four different in vitro tests', *Vet Parasitol*, 183: 103-8.

Kim, Y. W., M. J. Kim, B. Y. Chung, Y. Bang du, S. K. Lim, S. M. Choi, D. S. Lim, M. C. Cho, K. Yoon, H. S. Kim, K. B. Kim, Y. S. Kim, S. J. Kwack, and B. M. Lee.

2013. 'Safety evaluation and risk assessment of d-Limonene', *J Toxicol Environ Health B Crit Rev*, 16: 17-38.

Lamson, P. D., and C. B. Ward. 1932. 'The chemotherapy of helminth infestations', *J. Parasitol.*, 18: 173-99.

Le Hesran, J. Y., J. Akiana, H. M. Ndiaye el, M. Dia, P. Senghor, and L. Konate. 2004. 'Severe malaria attack is associated with high prevalence of Ascaris lumbricoides infection among children in rural Senegal', *Trans R Soc Trop Med Hyg*, 98: 397-9.

Lei, J., M. Leser, and E. Enan. 2010. 'Nematicidal activity of two monoterpenoids and SER-2 tyramine receptor of Caenorhabditis elegans', *Biochem Pharmacol*, 79: 1062-71.

Lemes, R. S., C. C. F. Alves, E. B. B. Estevam, M. B. Santiago, C. H. G. Martins, Tcld Santos, A. E. M. Crotti, and M. L. D. Miranda. 2018. 'Chemical composition and antibacterial activity of essential oils from Citrus aurantifolia leaves and fruit peel against oral pathogenic bacteria', *An Acad Bras Cienc*, 90: 1285-92.

Lota, M. L., D. de Rocca Serra, F. Tomi, C. Jacquemond, and J. Casanova. 2002. 'Volatile components of peel and leaf oils of lemon and lime species', *J Agric Food Chem*, 50: 796-805.

Lozon, Y., A. Sultan, S. J. Lansdell, T. Prytkova, B. Sadek, K. H. Yang, F. C. Howarth, N. S. Millar, and M. Oz. 2016. 'Inhibition of human alpha7 nicotinic acetylcholine receptors by cyclic monoterpene carveol', *Eur J Pharmacol*, 776: 44-51.

Macedo, I. T., C. M. Bevilaqua, L. M. de Oliveira, A. L. Camurca-Vasconcelos, S. Vieira Lda, F. R. Oliveira, E. M. Queiroz-Junior, B. G. Portela, R. S. Barros, and A. C. Chagas. 2009. 'Ovicidal and larvicidal activity in vitro of Eucalyptus globulus essential oils on Haemonchus contortus', *Rev Bras Parasitol Vet*, 18: 62-6.

Martin, R. J. 1997. 'Modes of action of anthelmintic drugs', *Vet J*, 154: 11-34.

Morgan, Eric, Johannes Charlier, Guy Hendrickx, Annibale Biggeri, Dolores Catalan, Georg von Samson-Himmelstjerna, Janina Demeler, Elizabeth Müller, Jan van Dijk, Fiona Kenyon, Philip Skuce, Johan Höglund, Padraig Kiely, Bonny van Ranst, Theo de Waal, Laura Rinaldi, Giuseppe Cringoli, Hubertus Hertzberg, Paul Torgerson, Adrian Wolstenholme, and Jozef Vercruysse. 2013. 'Global Change and Helminth Infections in Grazing Ruminants in Europe: Impacts, Trends and Sustainable Solutions', *Agriculture*, 3: 484.

Newman, D. J., and G. M. Cragg. 2016. 'Natural Products as Sources of New Drugs from 1981 to 2014', *J Nat Prod*, 79: 629-61.

Newman, D. J., G. M. Cragg, and K. M. Snader. 2000. 'The influence of natural products upon drug discovery', *Nat Prod Rep*, 17: 215-34.

Panella, N. A., M. C. Dolan, J. J. Karchesy, Y. Xiong, J. Peralta-Cruz, M. Khasawneh, J. A. Montenieri, and G. O. Maupin. 2005. 'Use of novel compounds for pest control: insecticidal and acaricidal activity of essential oil components from heartwood of Alaska yellow cedar', *J Med Entomol*, 42: 352-8.

Park, T. J., Y. S. Park, T. G. Lee, H. Ha, and K. T. Kim. 2003. 'Inhibition of acetylcholine-mediated effects by borneol', *Biochem Pharmacol*, 65: 83-90.

Park, T. J., H. K. Seo, B. J. Kang, and K. T. Kim. 2001. 'Noncompetitive inhibition by camphor of nicotinic acetylcholine receptors', *Biochem Pharmacol*, 61: 787-93.

Pessoa, L. M., S. M. Morais, C. M. Bevilaqua, and J. H. Luciano. 2002. 'Anthelmintic activity of essential oil of Ocimum gratissimum Linn. and eugenol against Haemonchus contortus', *Vet Parasitol*, 109: 59-63.

Petrovska, B. B. 2012. 'Historical review of medicinal plants' usage', *Pharmacogn Rev*, 6: 1-5.

Pirttila, T., G. Wilcock, L. Truyen, and C. V. Damaraju. 2004. 'Long-term efficacy and safety of galantamine in patients with mild-to-moderate Alzheimer's disease: multicenter trial', *Eur J Neurol*, 11: 734-41.

Rapior, Sylvie, Chantal Marion, Yves Pélissier, and Jean-Marie Bessière. 1997. 'Volatile Composition of Fourteen Species of Fresh Wild Mushrooms (Boletales)', *J. Essent Oil Res.*, 9: 231-34.

Raskin, I., and C. Ripoll. 2004. 'Can an apple a day keep the doctor away?', *Curr Pharm Des*, 10: 3419-29.

Robertson, A. P., and R. J. Martin. 2007. 'Ion-channels on parasite muscle: pharmacology and physiology', *Invert Neurosci*, 7: 209-17.

Roselli, M., M. S. Britti, I. Le Huerou-Luron, H. Marfaing, W. Y. Zhu, and E. Mengheri. 2007. 'Effect of different plant extracts and natural substances (PENS) against membrane damage induced by enterotoxigenic Escherichia coli K88 in pig intestinal cells', *Toxicol In Vitro*, 21: 224-9.

Samuelsson, G. 2004. *Drugs of Natural Origin: A Textbook of Pharmacognosy, 5th Edn.* (Swedish Pharmaceutical Press.: Stockholm).

Savioli, L., and M. Albonico. 2004. 'Soil-transmitted helminthiasis', *Nat Rev Microbiol*, 2: 618-9.

Squires, J. M., J. G. Foster, D. S. Lindsay, D. L. Caudell, and A. M. Zajac. 2010. 'Efficacy of an orange oil emulsion as an anthelmintic against Haemonchus contortus in gerbils (Meriones unguiculatus) and in sheep', *Vet Parasitol*, 172: 95-9.

Stammati, A., P. Bonsi, F. Zucco, R. Moezelaar, H. L. Alakomi, and A. von Wright. 1999. 'Toxicity of selected plant volatiles in microbial and mammalian short-term assays', *Food Chem Toxicol*, 37: 813-23.

Stephenson, L. S., M. C. Latham, and E. A. Ottesen. 2000. 'Malnutrition and parasitic helminth infections', *Parasitology*, 121 Suppl: S23-38.

Taman, A., and M. Azab. 2014. 'Present-day anthelmintics and perspectives on future new targets', *Parasitol Res*, 113: 2425-33.

Ton, H. T., A. E. Smart, B. L. Aguilar, T. T. Olson, K. J. Kellar, and G. P. Ahern. 2015. 'Menthol Enhances the Desensitization of Human alpha3beta4 Nicotinic Acetylcholine Receptors', *Mol Pharmacol*, 88: 256-64.

Tong, F., and J. R. Coats. 2012. 'Quantitative structure-activity relationships of monoterpenoid binding activities to the housefly GABA receptor', *Pest Manag Sci*, 68: 1122-9.

Tong, F., A. D. Gross, M. C. Dolan, and J. R. Coats. 2013. 'The phenolic monoterpenoid carvacrol inhibits the binding of nicotine to the housefly nicotinic acetylcholine receptor', *Pest Manag Sci*, 69: 775-80.

Trailović, S. M., D. S. Marjanovic, J. Nedeljkovic Trailovic, A. P. Robertson, and R. J. Martin. 2015. 'Interaction of carvacrol with the Ascaris suum nicotinic acetylcholine receptors and gamma-aminobutyric acid receptors, potential mechanism of antinematodal action', *Parasitol Res*, 114: 3059-68.

Vieira, A. J., F. P. Beserra, M. C. Souza, B. M. Totti, and A. L. Rozza. 2018. 'Limonene: Aroma of innovation in health and disease', *Chem Biol Interact*, 283: 97-106.

WHO. 2005. 'National Policy on Traditional Medicine and Regulation of Herbal Medicines- Report of a WHO Global Survey.', Geneva, Switzerland: WHO. http://apps.who.int/medicinedocs/en/d/Js7916e/.

———. 2018. 'Soil-transmitted helminth infections: Fact Sheet. http://www.who.int/en/news-room/fact-sheets/detail/soil-transmitted-helminth-infections [Accessed 20 February 2018]', Accessed 20 February. http://www.who.int/en/news-room/fact-sheets/detail/soil-transmitted-helminth-infections.

Williams, D. K., J. Wang, and R. L. Papke. 2011. 'Positive allosteric modulators as an approach to nicotinic acetylcholine receptor-targeted therapeutics: advantages and limitations', *Biochem Pharmacol*, 82: 915-30.

Zheng, F., A. P. Robertson, M. Abongwa, E. W. Yu, and R. J. Martin. 2016. 'The Ascaris suum nicotinic receptor, ACR-16, as a drug target: Four novel negative allosteric modulators from virtual screening', *Int J Parasitol Drugs Drug Resist*, 6: 60-73.

CHAPTER 6. GENERAL DISCUSSION

Anthelmintic chemotherapy relies heavily on a limited collection of antinematodal drugs. In veterinary field, multi-drug resistant nematodes that withstand all available classes of anthelmintics are increasingly common (Kaplan 2004; Wolstenholme et al. 2004; Taman and Azab 2014). This has raised concerns of drug resistance in human therapy and underscores the need for new strategies to combat these debilitating infections (Prichard et al. 2012; Geerts and Gryseels 2000). My doctoral research has identified different strategies to address the concerns of a depleted drug-discovery pipeline and drug resistance. We have described new anthelmintic drug targets and identified plant-based compounds that can be used in combination with current cholinomimetic drugs.

We have expressed and characterized a first of its kind, non-canonical homomeric nAChR formed by a non-alpha subunit, EAT-2, from the pharynx of *C. elegans*. In the free-living nematode, the receptor is required for cholinergic neurotransmission and regulates pharyngeal pumping, hence feeding behavior. We also characterized the homologous EAT-2 receptor from *A. suum* that had similar pharmacological profile to the cholinergic component of the *A. suum* pharynx. The pharyngeal nAChR from both the species has different pharmacological sensitivities from previously characterized nematode somatic muscle and vertebrate nAChRs. This receptor is “druggable” as it was not sensitive to existing cholinomimetic anthelmintics. A selective compound targeting this cation selective channel should lead to pharyngeal paralysis in worms, resulting in starvation or sluggish behavior in worms, and faster expulsion from the host. Thus, the EAT-2 nAChR meets the criteria for an anthelmintic target. It affects a vital function in

the worms, is conserved across species, and is pharmacologically diverse from the mammalian orthologues.

A novel approach towards antinematodal target discovery would be identification and validation of receptor-associated proteins in addition to new receptor targets (Maher et al. 2017). In the case of ionotropic receptors various auxiliary subunit proteins have been identified. These subunits physically associate with the ion channels and modulate their biological activity (Yan and Tomita 2012; Boulin et al. 2012; Greger, Watson, and Cull-Candy 2017). The association of auxiliary proteins with the receptor complex offers additional drug binding sites. Thus, targeting these interacting components can provide a new dimension for drug discovery. We have identified a novel auxiliary protein, EAT-18, which is required for the functional *in vitro* expression of the EAT-2 cation selective channels. We have demonstrated that EAT-18 physically interacts with the mature receptor and different homologues of the protein alter the pharmacological properties. As with *eat-2*, *eat-18* null mutants also result in pharyngeal defects. Thus, discovery of compounds that target EAT-18 should produce paralyzing effects on pharyngeal pumping behavior.

We have also characterized a homologue of the nematode ACR-16 nAChR subunit from *A. caninum.* Earlier studies have reported *A. suum* ACR-16 nAChRs to be a suitable target for the development of antinematodal drugs. In *C. elegans acr-16* is expressed in body wall muscles and encodes for nicotine-sensitive nAChRs (Touroutine et al. 2005; Richmond and Jorgensen 1999). Genetic screening in *C. elegans* has shown that the receptor regulates the locomotor behavior in combination with levamisole sensitive nAChR subunits (Touroutine et al. 2005; Li et al. 2014). While in *B. malayi* and

A. suum other tissue-related functions, including reproduction and digestion, have been suggested for the ACR-16 nAChR (Abongwa, Buxton, et al. 2016; Verma et al. 2017). *Aca*-ACR-16 shares 78 % identity with *A. suum* and *C. elegans* homologues. Based on our electrophysiology findings, the *Aca*-ACR-16 nAChR was not activated by many of the existing cholinomimetic anthelmintics (levamisole, pyrantel, morantel, bephenium and tribendimidine). Thus, the pharmacology of the receptor is distinct from the somatic muscle nematode nAChRs. Even though the protein sequence of the ACR-16 homologues is highly conserved, the ACR-16 homologue from *A. caninum* displayed some pharmacological differences from *Asu*-ACR-16. This can be attributed to variation of amino acids residues in the loops E and F. There is a need for new drug targets for hookworm infections. *A. caninum* ACR-16 is pharmacologically distinct from the previously characterized nematode nAChRs and may serve as a valid target site.

We have demonstrated the potential of three monoterpenoid compounds, namely, menthol, limonene oxide, and carvacrol for antinematodal therapy in combination with existing cholinergic compounds. Earlier studies have shown that carvacrol can act on GABA (gamma amino-butyric acid) and tyramine receptors. In our studies, carvacrol produced significant non-competitive inhibition on the levamisole sensitive nAChR from *O. dentatum* and nicotine sensitive nAChR from *A. suum*. Thus, this monoterpenoid phytocompound has the capability to bind to multiple types of pharmacological receptor which can impede the development of resistance. Limonene oxide produced non-competitive inhibition of the levamisole sensitive nAChRs and thus binds to a site distinct from ligand binding site. It may be possible to combine these compounds with an nAChR antagonist to produce a more potent effect. Menthol (0.1 μM and 10 μM)

potentiated acetylcholine and levamisole mediated responses in the levamisole sensitive nAChR. 0.1 μM menthol significantly potentiated the peak contractions produced by acetylcholine on *A. suum* somatic muscle strips. Positive allosteric modulators bind to a site distinct from the highly conserved ligand binding sites, which helps with better selectivity profile and reduced potential for development of resistance. Menthol behaves as a positive allosteric modulator of the levamisole sensitive nAChRs. Combination of menthol with cholinomimetic drugs like pyrantel or levamisole may produce a more potent anthelmintic effect.

6.1 Future directions

There are a number of experiments that can be done to further validate the EAT-2 nAChR and associated auxiliary protein, EAT-18, as a potential drug target. We would recommend expressing the EAT-2 nAChR in cell lines or yeast cells which will facilitate high-throughput drug screening. This will allow target-based screening of chemical libraries to identify selective drugs for the receptor. These experiments will also assist with confirming the pharmacological diversity of the receptor by screening for compounds that are specific for mammalian orthologues. Additionally, development of three-dimensional models for the protein will help in structure-based virtual screening of potential anthelmintic compounds. We also recommend identification and cloning of putative homologues from EAT-2 and EAT-18 from parasites of human medical relevance for further validation across nematode species.

We would recommend similar expression and drug screening experiments for *A. caninum* ACR-16 nAChRs to identify selective anthelmintics for hookworm infections. Additionally, RNAi techniques and genetic screens can be used to understand the role of

the ACR-16 homologue in the hookworms. RT-PCR analysis of various hookworm tissues will also help in determining the expression pattern and physiological relevance of the nAChR.

The potential antinematodal monoterpenoid compounds identified should be evaluated for their efficacy and toxicity through various *in vitro* and *in vivo* assays. The combination of these compounds with various cholinergic compounds should also be evaluated.

REFERENCES

Abongwa, M., S. K. Buxton, E. Courtot, C. L. Charvet, C. Neveu, C. J. McCoy, S. Verma, A. P. Robertson, and R. J. Martin. 2016. 'Pharmacological profile of Ascaris suum ACR-16, a new homomeric nicotinic acetylcholine receptor widely distributed in Ascaris tissues', *Br J Pharmacol*, 173: 2463-77.

Abongwa, M., R. J. Martin, and A. P. Robertson. 2017. 'A brief review on the mode of action of antinematodal drugs.', *Acta veterinaria*, 67: 137-52.

Aceves, J., D. Erlij, and R. Martínez-Marañón. 1970. 'The mechanism of the paralysing action of tetramisole on Ascaris somatic muscle', *British Journal of Pharmacology*, 38: 602-07.

Adelman, J. P. 1995. 'Proteins that interact with the pore-forming subunits of voltage-gated ion channels', *Current Opinion in Neurobiology*, 5: 286-95.

Akuthota, P., and P F. Weller. 2012. 'Eosinophilic Pneumonias', *Clinical Microbiology Reviews*, 25: 649.

Albertson, D. G., and J. N. Thomson. 1976. 'The pharynx of Caenorhabditis elegans', *Philos Trans R Soc Lond B Biol Sci*, 275: 299-325.

Albonico, M., D. W. T. Crompton, and L. Savioli. 1999. 'Control Strategies for Human Intestinal Nematode Infections.' in J. R. Baker, R. Muller and D. Rollinson (eds.), *Advances in Parasitology* (Academic Press).

Albonico, M., R. J. Stoltzfus, L. Savioli, J. M. Tielsch, H. M. Chwaya, E. Ercole, and G. Cancrini. 1998. 'Epidemiological evidence for a differential effect of hookworm species, Ancylostoma duodenale or Necator americanus, on iron status of children', *Int J Epidemiol*, 27: 530-7.

Alexander, A. G., V. Marfil, and C. Li. 2014. 'Use of Caenorhabditis elegans as a model to study Alzheimer's disease and other neurodegenerative diseases', *Frontiers in Genetics*, 5.

Alfonso, A., K. Grundahl, J. S. Duerr, H. P. Han, and J. B. Rand. 1993. 'The Caenorhabditis elegans unc-17 gene: a putative vesicular acetylcholine transporter', *Science*, 261: 617-19.

Alkema, M. J., M. Hunter-Ensor, N. Ringstad, and H. R. Horvitz. 2005. 'Tyramine Functions Independently of Octopamine in the Caenorhabditis elegans Nervous System', *Neuron*, 46: 247-60.

Altun, Z. F., B. Chen, Z. W. Wang, and D. H. Hall. 2009. 'High resolution map of Caenorhabditis elegans gap junction proteins', *Dev Dyn*, 238: 1936-50.

Alum, A., J. R. Rubino, and M. K. Ijaz. 2010. 'The global war against intestinal parasites—should we use a holistic approach?', *International Journal of Infectious Diseases*, 14: e732-e38.

Anderson, R. C. 1992. *Nematode parasites of vertebrates : their development and transmission / R.C. Anderson* (Wallingford, Oxon, UK : C.A.B.International: Wallingford, Oxon, UK).

Andre, W. P. P., W. L. C. Ribeiro, G. S. Cavalcante, J. M. L. dos Santos, I. T. F. Macedo, H. C. B. de Paula, R. M. de Freitas, S. M. de Morais, J. V. de Melo, and C. M. L. Bevilaqua. 2016. 'Comparative efficacy and toxic effects of carvacryl acetate and carvacrol on sheep gastrointestinal nematodes and mice', *Veterinary Parasitology*, 218: 52-58.

Andrés, M., A. González-Coloma, J. Sanz, J. Burillo, and P. Sainz. 2012. 'Nematicidal activity of essential oils: a review', *Phytochem Rev*, 11: 371-90.

Anquez-Traxler, Christelle. 2011. 'The Legal and Regulatory Framework of Herbal Medicinal Products in the European Union: A Focus on the Traditional Herbal Medicines Category', *Drug Information Journal*, 45: 15-23.

Arena, J. P., K. K. Liu, P. S. Paress, J. M. Schaeffer, and D. F. Cully. 1992. 'Expression of a glutamate-activated chloride current in Xenopus oocytes injected with Caenorhabditis elegans RNA: evidence for modulation by avermectin', *Brain Res Mol Brain Res*, 15: 339-48.

Arias, H. R. 1997. 'Topology of ligand binding sites on the nicotinic acetylcholine receptor', *Brain Research Reviews*, 25: 133-91.

Aubry, M. L., P. Cowell, M. J. Davey, and S. Shevde. 1970. 'Aspects of the pharmacology of a new anthelmintic: pyrantel.', *British Journal of Pharmacology*, 38: 332-44.

Avato, P., S. Laquale, M. Argentieri, A. Lamiri, V. Radicci, and T. D'Addabbo. 2017. 'Nematicidal activity of essential oils from aromatic plants of Morocco', *J Pest Sci*, 90: 711-22.

Avery, L. 1993a. 'The genetics of feeding in Caenorhabditis elegans', *Genetics*, 133: 897-917.

———. 1993b. 'Motor neuron M3 controls pharyngeal muscle relaxation timing in Caenorhabditis elegans', *J Exp Biol*, 175: 283-97.

Avery, L., C. I. Bargmann, and H. R. Horvitz. 1993. 'The Caenorhabditis elegans unc-31 gene affects multiple nervous system-controlled functions', *Genetics*, 134: 455-64.

Avery, L., and H. R. Horvitz. 1987. 'A cell that dies during wild-type C. elegans development can function as a neuron in a ced-3 mutant', *Cell*, 51: 1071-8.

———. 1989. 'Pharyngeal pumping continues after laser killing of the pharyngeal nervous system of C. elegans', *Neuron*, 3: 473-85.

———. 1990. 'Effects of starvation and neuroactive drugs on feeding in Caenorhabditis elegans', *J Exp Zool*, 253: 263-70.

Avery, L., D. Raizen, and S. Lockery. 1995. 'Electrophysiological methods', *Methods Cell Biol*, 48: 251-69.

Avery, L., and B. B. Shtonda. 2003. 'Food transport in the C. elegans pharynx', *J Exp Biol*, 206: 2441-57.

Avery, L., and J. H. Thomas. 1997. 'Feeding and Defecation.' in Riddle D. L., Blumenthal T., Meyer B. J. and et al. (eds.), *C. elegans II* (Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York).

Awasthi, S., D. A. P. Bundy, and L. Savioli. 2003. 'Helminthic infections', *BMJ (Clinical research ed.)*, 327: 431-33.

Ballivet, M., C. Alliod, S. Bertrand, and D. Bertrand. 1996. 'Nicotinic Acetylcholine Receptors in the NematodeCaenorhabditis elegans', *Journal of Molecular Biology*, 258: 261-69.

Bargmann, C. I. 1993. 'Genetic and cellular analysis of behavior in C. elegans', *Annu Rev Neurosci*, 16: 47-71.

———. 1998. 'Neurobiology of the Caenorhabditis elegans Genome', *Science*, 282: 2028.

Barros, A. G. d. A., J. C. Bridi, B. R. de Souza, C. de Castro Júnior, K. C. de Lima Torres, L. Malard, A. Jorio, D. M. de Miranda, K. Ashrafi, and M. A. Romano-Silva. 2014. 'Dopamine Signaling Regulates Fat Content through β-Oxidation in Caenorhabditis elegans', *PLOS ONE*, 9: e85874.

Bartsch, S. M., P. J. Hotez, L. Asti, K. M. Zapf, M. Elena Bottazzi, D. J. Diemert, and Br. Y. Lee. 2016. 'The Global Economic and Health Burden of Human Hookworm Infection', *PLOS Neglected Tropical Diseases*, 10: e0004922.

Basyoni, M. M. A., and E. M. A. Rizk. 2016. 'Nematodes ultrastructure: complex systems and processes', *Journal of parasitic diseases : official organ of the Indian Society for Parasitology*, 40: 1130-40.

Bennet-Clark, H.C. 1976. 'Mechanics of nematode feeding.' in N. A. Croll (ed.), *The Organization of Nematodes* (Academic Press, London, New Yor, San Fransisco).

Bennett, M. R. 2000. 'The concept of transmitter receptors: 100 years on', *Neuropharmacology*, 39: 523-46.

Bethony, J., S. Brooker, M. Albonico, S. M. Geiger, A. Loukas, D. Diemert, and P. J. Hotez. 2006. 'Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm', *The Lancet*, 367: 1521-32.

Bethony, J., J. Chen, S. Lin, S. Xiao, B. Zhan, S. Li, H. Xue, F. Xing, D. Humphries, W. Yan, G. Chen, V. Foster, J. M. Hawdon, and P. J. Hotez. 2002. 'Emerging patterns of hookworm infection: influence of aging on the intensity of Necator infection in Hainan Province, People's Republic of China', *Clin Infect Dis*, 35: 1336-44.

Bird, A. F. 1991. *The structure of nematodes / Alan F. Bird, Jean Bird* (San Diego : Academic Press: San Diego).

Bissinger, B. W., C. T. Knox, S. M. Mitchell, and R. M. Kaplan. 2014. 'Activity of Plant-Based Compounds on Anthelmintic-Resistant Caenorhabditis elegans.' in, *Biopesticides: State of the Art and Future Opportunities* (American Chemical Society).

Blackwell, V., and F. Vega-Lopez. 2001. 'Cutaneous larva migrans: clinical features and management of 44 cases presenting in the returning traveller', *The British journal of dermatology*, 145: 434.

Blanchard, Alexandra, Fabrice Guégnard, Claude L. Charvet, Anna Crisford, Elise Courtot, Christine Sauvé, Abdallah Harmache, Thomas Duguet, Vincent O'Connor, Philippe Castagnone-Sereno, Barbara Reaves, Adrian J. Wolstenholme, Robin N. Beech, Lindy Holden-Dye, and Cedric Neveu. 2018. 'Deciphering the molecular determinants of cholinergic anthelmintic sensitivity in nematodes: When novel functional validation approaches highlight major differences between the model Caenorhabditis elegans and parasitic species', *PLOS Pathogens*, 14: e1006996-e96.

Blaxter, M. L., P. De Ley, J. R. Garey, L. X. Liu, P. Scheldeman, A. Vierstraete, J. R. Vanfleteren, L. Y. Mackey, M. Dorris, L. M. Frisse, J. T. Vida, and W. K. Thomas. 1998. 'A molecular evolutionary framework for the phylum Nematoda', *Nature*, 392: 71.

Blotkamp, J., H. P. Krepel, V. Kumar, S. Baeta, J. M. Van't Noordende, and A. M. Polderman. 1993. 'Observations on the morphology of adults and larval stages of Oesophagostomum sp. isolated from man in northern Togo and Ghana', *Journal of Helminthology*, 67: 49-61.

Bogers, J. J., P. A. Storey, G. Faile, E. Hewitt, L. Yelifari, A. Polderman, and E. A. Van Marck. 2001. 'Human oesophagostomiasis: a histomorphometric study of 13 new cases in northern Ghana', *Virchows Arch*, 439: 21-6.

Borchardt, J. K. 2002. 'The Beginnings of Drug Therapy: Ancient Mesopotamian Medicine', *Drug News Perspect*, 15: 187-92.

Bosch, F., and L. Rosich. 2008. 'The contributions of Paul Ehrlich to pharmacology: a tribute on the occasion of the centenary of his Nobel Prize', *Pharmacology*, 82: 171-79.

Boulin, T., A. Fauvin, C. L. Charvet, J. Cortet, J. Cabaret, J. L. Bessereau, and C. Neveu. 2011. 'Functional reconstitution of Haemonchus contortus acetylcholine receptors in Xenopus oocytes provides mechanistic insights into levamisole resistance', *British Journal of Pharmacology*, 164: 1421-32.

Boulin, T., G. Rapti, L. Briseño-Roa, C. Stigloher, J. E. Richmond, P. Paoletti, and J.-L. Bessereau. 2012. 'Positive modulation of a Cys-loop acetylcholine receptor by an auxiliary transmembrane subunit', *Nature Neuroscience*, 15: 1374.

Boulin, Thomas, Marc Gielen, Janet E. Richmond, Daniel C. Williams, Pierre Paoletti, and Jean-Louis Bessereau. 2008. 'Eight genes are required for functional reconstitution of the Caenorhabditis elegans levamisole-sensitive acetylcholine receptor', *Proceedings of the National Academy of Sciences of the United States of America*, 105: 18590-95.

Brejc, K., W. J. van Dijk, R. V. Klaassen, M. Schuurmans, J. van der Oost, A. B. Smit, and T.K. Sixma. 2001. 'Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors', *Nature*, 411: 269-76.

Brenner, S. 1973. 'The genetics of behaviour', *Br Med Bull*, 29: 269-71.

Briggs, C. A., D. G. McKenna, and M. Piattina-kaplan. 1995. 'Human α 7 nicotinic acetylcholine receptor responses to novel ligands', *Neuropharmacology*, 34: 583-90.

Britton, Collette, and Linda Murray. 2006. 'Using Caenorhabditis elegans for functional analysis of genes of parasitic nematodes', *International Journal for Parasitology*, 36: 651-59.

Brockie, P. J., J. E. Mellem, T. Hills, D. M. Madsen, and A. V. Maricq. 2001. 'The C. elegans glutamate receptor subunit NMR-1 is required for slow NMDA-activated currents that regulate reversal frequency during locomotion', *Neuron*, 31: 617-30.

Brooker, S., P. J. Hotez, and D. A. Bundy. 2008. 'Hookworm-related anaemia among pregnant women: a systematic review', *PLoS Negl Trop Dis*, 2: e291.

Brown, L. A., A. K. Jones, S. D. Buckingham, C. J. Mee, and D. B. Sattelle. 2006. 'Contributions from Caenorhabditis elegans functional genetics to antiparasitic drug target identification and validation: Nicotinic acetylcholine receptors, a case study', *International Journal for Parasitology*, 36: 617-24.

Brownlee, D. J. A., I. Fairweather, L. Holden-Dye, and R. J. Walker. 1996. 'Nematode neuropeptides: Localization, isolation and functions', *Parasitology Today*, 12: 343-51.

Brownlee, D. J., I. Fairweather, and C. F. Johnston. 1993. 'Immunocytochemical demonstration of neuropeptides in the peripheral nervous system of the roundworm Ascaris suum (Nematoda, Ascaroidea)', *Parasitol Res*, 79: 302-8.

Brownlee, D. J., L. Holden-Dye, and R. J. Walker. 1997. 'Actions of the anthelmintic ivermectin on the pharyngeal muscle of the parasitic nematode, Ascaris suum', *Parasitology*, 115 (Pt 5): 553-61.

Brownlee, D. J., L. Holden-Dye, R. J. Walker, and I. Fairweather. 1995. 'The pharynx of the nematode Ascaris suum: structure and function', *Acta Biol Hung*, 46: 195-204.

Bruggmann, D., K. S. Lips, U. Pfeil, R. V. Haberberger, and W. Kummer. 2002. 'Multiple nicotinic acetylcholine receptor alpha-subunits are expressed in the arterial system of the rat', *Histochem Cell Biol*, 118: 441-7.

Brusca, R. C., and G. J. B. Brusca. 1990. *Invertebrates* (Sunderland, Mass. : Sinauer Associates: Sunderland, Mass.).

Buckingham, S. D., F. A. Partridge, and D. B. Sattelle. 2014. 'Automated, high-throughput, motility analysis in Caenorhabditis elegans and parasitic nematodes: Applications in the search for new anthelmintics', *Int J Parasitol Drugs Drug Resist*, 4: 226-32.

Bundy, D. A., M. S. Chan, and L. Savioli. 1995. 'Hookworm infection in pregnancy', *Trans R Soc Trop Med Hyg*, 89: 521-2.

Bürglin, Thomas R., Edgar Lobos, and Mark L. Blaxter. 1998. 'Caenorhabditis elegans as a model for parasitic nematodes', *International Journal for Parasitology*, 28: 395-411.

Burns, A. R., G. M. Luciani, G. Musso, R. Bagg, M. Yeo, Y. Zhang, L. Rajendran, J. Glavin, R. Hunter, E. Redman, S. Stasiuk, M. Schertzberg, G. Angus McQuibban, C. R. Caffrey, S. R. Cutler, M. Tyers, G. Giaever, C. Nislow, A. G. Fraser, C. A. MacRae, J. Gilleard, and P. J. Roy. 2015. 'Caenorhabditis elegans is a useful model for anthelmintic discovery', *Nat Commun*, 6: 7485.

Butler, M. S. 2004. 'The role of natural product chemistry in drug discovery', *J Nat Prod*, 67: 2141-53.

Buxton, S. K., C. L. Charvet, C. Neveu, J. Cabaret, J. Cortet, N. Peineau, M. Abongwa, E. Courtot, A. P. Robertson, and R. J. Martin. 2014. 'Investigation of acetylcholine receptor diversity in a nematode parasite leads to characterization of tribendimidine- and derquantel-sensitive nAChRs', *PLOS Pathogens*, 10: e1003870-e70.

Byerly, L., and M. O. Masuda. 1979. 'Voltage-clamp analysis of the potassium current that produces a negative-going action potential in Ascaris muscle', *The Journal of Physiology*, 288: 263-84.

Calapai, G. 2008. 'European legislation on herbal medicines: a look into the future', *Drug Saf*, 31: 428-31.

Camurca-Vasconcelos, A. L., C. M. Bevilaqua, S. M. Morais, M. V. Maciel, C. T. Costa, I. T. Macedo, L. M. Oliveira, R. R. Braga, R. A. Silva, and L. S. Vieira. 2007. 'Anthelmintic activity of Croton zehntneri and Lippia sidoides essential oils', *Vet Parasitol*, 148: 288-94.

Carre-Pierrat, M., D. Baillie, R. Johnsen, R. Hyde, A. Hart, L. Granger, and L. Ségalat. 2006. 'Characterization of the Caenorhabditis elegans G protein-coupled serotonin receptors', *Invertebrate Neuroscience*, 6: 189-205.

Castro, G. A. 1996. 'Helminths: Structure, Classification, Growth, and Development.' in S. Baron (ed.), *Medical Microbiology* (University of Texas Medical Branch at Galveston.: Galveston (TX)).

Cetin, H., J. E. Cilek, L. Aydin, and A. Yanikoglu. 2009. 'Acaricidal effects of the essential oil of Origanum minutiflorum (Lamiaceae) against Rhipicephalus turanicus (Acari: Ixodidae)', *Vet Parasitol*, 160: 359-61.

Chalfie, Martin. 1984. 'Neuronal development in Caenorhabditis elegans', *Trends in Neurosciences*, 7: 197-202.

Chandler, A. C. 1949. *Introduction to parasitology, with special reference to the parasites of man* (New York, J. Wiley: New York).

Changeux, J.-P. 2012. 'The nicotinic acetylcholine receptor: the founding father of the pentameric ligand-gated ion channel superfamily', *The Journal of biological chemistry*, 287: 40207-15.

Changeux, J.-P., and S. J. Edelstein. 2006. 'Allosteric receptors after 30 years', *Rendiconti Lincei*, 17: 59.

Changeux, J.-P., M. Kasai, and C.-Y. Lee. 1970. 'Use of a Snake Venom Toxin to Characterize the Cholinergic Receptor Protein', *Proceedings of the National Academy of Sciences*, 67: 1241.

Charlton, A. 2004. 'Medicinal uses of tobacco in history', *Journal of the Royal Society of Medicine*, 97: 292-96.

Charvet, Claude L., Alan P. Robertson, Jacques Cabaret, Richard J. Martin, and Cédric Neveu. 2012. 'Selective effect of the anthelmintic bephenium on Haemonchus contortus levamisole-sensitive acetylcholine receptors', *Invertebrate neuroscience : IN*, 12: 43-51.

Chen, L., D. M. Chetkovich, R. S. Petralia, N. T. Sweeney, Y. Kawasaki, R. J. Wenthold, D. S. Bredt, and R. A. Nicoll. 2000. 'Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms', *Nature*, 408: 936.

Chiang, J.-T. A., M. Steciuk, B. Shtonda, and L. Avery. 2006. 'Evolution of pharyngeal behaviors and neuronal functions in free-living soil nematodes', *Journal of Experimental Biology*, 209: 1859-73.

Claus, Paul-Emile, Anne-Sophie Ceuppens, Mike Cool, and Gudrun Alliet. 2018. *Ascaris lumbricoides: challenges in diagnosis, treatment and prevention strategies in a European refugee camp*.

Cleaveland, S., M. K. Laurenson, and L. H. Taylor. 2001. 'Diseases of humans and their domestic mammals: pathogen characteristics, host range and the risk of emergence', *Philos Trans R Soc Lond B Biol Sci*, 356: 991-9.

Clementi, Francesco, Diego Fornasari, and Cecilia Gotti. 2000. 'Neuronal nicotinic receptors, important new players in brain function', *European Journal of Pharmacology*, 393: 3-10.

Cockburn, T. A., E. Cockburn, and T. A. Reyman. 1998. *Mummies, Disease and Ancient Cultures* (Cambridge University Press: Cambridge).

Consortium, C. elegans Sequencing. 1998. 'Genome sequence of the nematode C. elegans: a platform for investigating biology', *Science*, 282: 2012-8.

Cook, A. , C. J. Franks, and L. Holden-Dye. 2006. "Electrophysiological recordings from the pharynx." In *WormBook*, edited by William J. Schafer. The C. elegans Research Community, WormBook.

Cook, A., N. Aptel, V. Portillo, E. Siney, R. Sihota, L. Holden-Dye, and A. J. Wolstenholme. 2006. 'Caenorhabditis elegans ivermectin receptors regulate locomotor behaviour and are functional orthologues of Haemonchus contortus receptors', *Molecular and Biochemical Parasitology*, 147: 118-25.

Coombs, I., and D.W.T. Crompton. 1992. *A guide to human helminths T* (Taylor and Francis: London).

Cordero-Erausquin, M., L. M. Marubio, R. Klink, and J. P. Changeux. 2000. 'Nicotinic receptor function: new perspectives from knockout mice', *Trends Pharmacol Sci*, 21: 211-7.

Corringer, Pierre-Jean, Nicolas Le Novère, and Jean-Pierre Changeux. 2000. 'Nicotinic Receptors at the Amino Acid Level', *Annual Review of Pharmacology and Toxicology*, 40: 431-58.

Corsi, A. K. 2006. 'A biochemist's guide to Caenorhabditis elegans', *Anal Biochem*, 359: 1-17.

Coskun, S., O. Girisgin, M. Kurkcuoglu, H. Malyer, A. O. Girisgin, N. Kirimer, and K. H. Baser. 2008. 'Acaricidal efficacy of Origanum onites L. essential oil against Rhipicephalus turanicus (Ixodidae)', *Parasitol Res*, 103: 259-61.

Cousin, M. T. 2013. 'History of anaesthesia: who discovered the neuromuscular junction? The opposing views of Claude Bernard and Alfred Vulpian', *Eur J Anaesthesiol*, 30: 1-4.

Couturier, Sabine, Daniel Bertrand, Jean-Marc Matter, Maria-Clemencia Hernandez, Sonia Bertrand, Neil Millar, Soledad Valera, Thomas Barkas, and Marc Ballivet. 1990. 'A neuronal nicotinic acetylcholine receptor subunit (α7) is developmentally regulated and forms a homo-oligomeric channel blocked by α-BTX', *Neuron*, 5: 847-56.

Cox, F. E. G. 2002. 'History of human parasitology', *Clinical Microbiology Reviews*, 15: 595-612.

Croll, N. A. 1975a. 'Components and patterns in the behaviour of the nematode Caenorhabditis elegans', *Journal of Zoology*, 176: 159-76.

———. 1975b. 'Indolealkylamines in the coordination of nematode behavioral activities', *Can J Zool*, 53: 894-903.

Croll, N. A., and J. M. Smith. 1978. 'Integrated behaviour in the feeding phase of Caenorhabditis elegans (Nematoda)', *Journal of Zoology*, 184: 507-17.

Crompton, D. W. T. 2001. 'Ascaris and ascariasis.' in, *Advances in Parasitology* (Academic Press).

Culetto, E., H. A. Baylis, J. E. Richmond, A. K. Jones, J. T. Fleming, M. D. Squire, J. A. Lewis, and D. B. Sattelle. 2004. 'The Caenorhabditis elegans unc-63 Gene Encodes a Levamisole-sensitive Nicotinic Acetylcholine Receptor α Subunit', *Journal of Biological Chemistry*, 279: 42476-83.

Cully, D. F., D. K. Vassilatis, K. K. Liu, P. S. Paress, L. H. T. Van der Ploeg, J. M. Schaeffer, and J. P. Arena. 1994. 'Cloning of an avermectin-sensitive glutamate-gated chloride channel from Caenorhabditis elegans', *Nature*, 371: 707-11.

Dale, V. M., and R. J. Martin. 1995. 'Oxantel-activated single channel currents in the muscle membrane of Ascaris suum', *Parasitology*, 110 (Pt 4): 437-48.

Dallière, N., L. Holden-Dye, J. Dillon, V. O'Connor, and R. J. Walker. 2017. "Caenorhabditis elegans Feeding Behaviors." In.: Oxford University Press.

Dani, John A. 2015. 'Neuronal Nicotinic Acetylcholine Receptor Structure and Function and Response to Nicotine', *International review of neurobiology*, 124: 3-19.

Dani, John A., Daoyun Ji, and Fu-Ming Zhou. 2001. 'Synaptic Plasticity and Nicotine Addiction', *Neuron*, 31: 349-52.

de Bono, M. 2003. 'Molecular approaches to aggregation behavior and social attachment', *J Neurobiol*, 54: 78-92.

de Silva, N. R., S. J. Brooker, P. J. Hotez, A. Montresor, D. Engels, and L. Savioli. 2003. 'Soil-transmitted helminth infections: updating the global picture', *Trends in Parasitology*, 19: 547-51.

De Silva, N. R., H. L. Guyatt, and D. A. P. Bundy. 1997. 'Worm burden in intestinal obstruction caused by Ascaris lumbricoides', *Tropical Medicine & International Health*, 2: 189-90.

de Vlas, Sake J., Wilma A. Stolk, Epke A. le Rutte, Jan A. C. Hontelez, Roel Bakker, David J. Blok, Rui Cai, Tanja A. J. Houweling, Margarete C. Kulik, Edeltraud J. Lenk, Marianne Luyendijk, Suzette M. Matthijsse, William K. Redekop, Inge Wagenaar, Julie Jacobson, Nico J. D. Nagelkerke, and Jan H. Richardus. 2016. 'Concerted Efforts to Control or Eliminate Neglected Tropical Diseases: How Much Health Will Be Gained?', *PLOS Neglected Tropical Diseases*, 10: e0004386.

DeBell, J. T. 1963. 'Electrpphysiology of the somatic muscle cells of Ascaris lumbricoides', *Journal of Cellular and Comparative Physiology*, 62: 159-77.

del Castillo, J., and T. Morales. 1967a. 'The Electrical and Mechanical Activity of the Esophageal Cell of Ascaris lumbricoides', *J. gen. Physiol.*, 50: 603.

———. 1967b. 'Extracellular Action Potentials Recorded from the Interior of the Giant Esophageal Cell of *Ascaris*', *J. gen. Physiol.*, 50: 631.

del Castillo, J., A. Rivera, S. Solorzano, and J. Serrato. 1989. 'Some aspects of the neuromuscular system of Ascaris', *Q J Exp Physiol*, 74: 1071-87.

Dent, J. A., M. W. Davis, and L. Avery. 1997. 'avr-15 encodes a chloride channel subunit that mediates inhibitory glutamatergic neurotransmission and ivermectin sensitivity in Caenorhabditis elegans', *The EMBO Journal*, 16: 5867-79.

Dent, J. A., M. M. Smith, D. K. Vassilatis, and L. Avery. 2000. 'The genetics of ivermectin resistance in Caenorhabditis elegans', *Proc Natl Acad Sci U S A*, 97: 2674-9.

Despommier, D. D., D. O. Griffin, R. W. Gwadz, P. J. Hotez, and C. A. Knirsch. 2017. *Parasitic Diseases 6th Edition* (Parsites without Borders, Inc. NY: New York).

Dieterich, C., and R. J. Sommer. 2009. 'How to become a parasite - lessons from the genomes of nematodes', *Trends Genet*, 25: 203-9.

Dold, Christina, and Celia V. Holland. 2011. 'Ascaris and ascariasis', *Microbes and Infection*, 13: 632-37.

Doncaster, C. C. 1962. 'Nematode Feeding Mechanisms. 1. Observations On Rhabditis and Pelodera', 8: 313.

Driscoll, M., E. Dean, E. Reilly, E. Bergholz, and M. Chalfie. 1989. 'Genetic and molecular analysis of a Caenorhabditis elegans beta-tubulin that conveys benzimidazole sensitivity', *J Cell Biol*, 109: 2993-3003.

Duguet, T. B., C. L. Charvet, S. G. Forrester, C. M. Wever, J. A. Dent, C. Neveu, and R. N. Beech. 2016. 'Recent Duplication and Functional Divergence in Parasitic Nematode Levamisole-Sensitive Acetylcholine Receptors', *PLOS Neglected Tropical Diseases*, 10: e0004826-e26.

Eimer, S., A. Gottschalk, M. Hengartner, H. R. Horvitz, J. Richmond, W. R. Schafer, and J. L. Bessereau. 2007. 'Regulation of nicotinic receptor trafficking by the transmembrane Golgi protein UNC-50', *EMBO J*, 26: 4313-23.

Elgoyhen, Ana B., David S. Johnson, Jim Boulter, Douglas E. Vetter, and Stephen Heinemann. 1994. 'α9: An acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells', *Cell*, 79: 705-15.

Enan, E. E. 2001. 'Insecticidal activity of essential oils: octopaminergic sites of action', *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 130: 325-37.

———. 2005. 'Molecular and pharmacological analysis of an octopamine receptor from American cockroach and fruit fly in response to plant essential oils', *Arch Insect Biochem Physiol*, 59: 161-71.

Ferreira, L. F. , A. J. G. Araújo, U. E. C. Confaloneiri, M. Chame, and B. M. Ribeiro. 1987. 'Encontro de ovos de ancilostomideos en coprolitos humano datados de 7230 +− 80 años, Piaui, Brasil.', *An. Acad. Bras. Cienc.*, 59: 280-81.

Ferreira, L. F., A. J. de Araujo, and U. E. Confalonieri. 1980. 'The finding of eggs and larvae of parasitic helminths in archaeological material from Unai, Minas Gerais, Brazil', *Trans R Soc Trop Med Hyg*, 74: 798-800.

———. 1983. 'The finding of helminth eggs in a Brazilian mummy', *Trans R Soc Trop Med Hyg*, 77: 65-7.

Ferrell, J. A. 1914. *The Rural School and Hookworm Disease* (Government Printing Office, : Washington D.C).

Fetterer, R. H., and M. L. Rhoads. 1993. 'Biochemistry of the nematode cuticle: relevance to parasitic nematodes of livestock', *Vet Parasitol*, 46: 103-11.

Fetterer, R. H., and M. Wasiuta. 1987. 'Ascaris suum: partial isolation and characterization of hypodermis from the adult female', *Exp Parasitol*, 63: 312-8.

Fitzsimmons, Colin, Franco Falcone, and David Dunne. 2014. 'Helminth Allergens, Parasite-Specific IgE, and Its Protective Role in Human Immunity', *Frontiers in Immunology*, 5.

Fleming, J. T., M. D. Squire, T. M. Barnes, C. Tornoe, K. Matsuda, J. Ahnn, A. Fire, J. E. Sulston, E. A. Barnard, D. B. Sattelle, and J. A. Lewis. 1997. 'Caenorhabditis elegans levamisole resistance genes lev-1, unc-29, and unc-38 encode functional nicotinic acetylcholine receptor subunits', *J Neurosci*, 17: 5843-57.

Franks, Christopher J., Lindy Holden-Dye, Kathryn Bull, Sarah Luedtke, and Robert J. Walker. 2006. 'Anatomy, physiology and pharmacology of Caenorhabditis elegans pharynx: a model to define gene function in a simple neural system', *Invertebrate Neuroscience*, 6: 105-22.

Galaz, P., R. Barra, H. Figueroa, and T. Mariqueo. 2015. 'Advances in the pharmacology of lGICs auxiliary subunits', *Pharmacological Research*, 101: 65-73.

Galzi, J. l, and J. p Changeux. 1995. 'Neuronal nicotinic receptors: Molecular organization and regulations', *Neuropharmacology*, 34: 563-82.

Galzi, J. L., D. Bertrand, A. Devillers-Thiery, F. Revah, S. Bertrand, and J. P. Changeux. 1991. 'Functional significance of aromatic amino acids from three peptide loops of the alpha 7 neuronal nicotinic receptor site investigated by site-directed mutagenesis', *FEBS Lett*, 294: 198-202.

Gardner, S. L. 2013. 'Worms, Nematoda.' in Simon A. Levin (ed.), *Encyclopedia of Biodiversity (Second Edition)* (Academic Press: Waltham).

Geary, T. G., and D. P. Thompson. 2001. 'Caenorhabditis elegans: how good a model for veterinary parasites?', *Vet Parasitol*, 101: 371-86.

Geerts, S., and B. Gryseels. 2000. 'Drug resistance in human helminths: current situation and lessons from livestock', *Clin Microbiol Rev*, 13: 207-22.

Gerzanich, V., R. Anand, and J. Lindstrom. 1994. 'Homomers of alpha 8 and alpha 7 subunits of nicotinic receptors exhibit similar channel but contrasting binding site properties', *Molecular Pharmacology*, 45: 212.

Ghai, Ria R., Colin A. Chapman, Patrick A. Omeja, T. Jonathan Davies, and Tony L. Goldberg. 2014. 'Nodule worm infection in humans and wild primates in Uganda: cryptic species in a newly identified region of human transmission', *PLOS Neglected Tropical Diseases*, 8: e2641-e41.

Gigase, P., S. Baeta, V. Kumar, and J. Brandt. 1987. 'Frequency of Symptomatic Human Oeophagostomiastis (Helminthoma) in Northern Togo.' in S. Geerts, V. Kumar and J. Brandt (eds.), *Helminth Zoonoses* (Springer Netherlands: Dordrecht).

Goldschmidt, R. 1904. 'Der Chromidialapparat Lebhaft Funktionierender Gewebszellen', *Zool. J.*, 21: 41-140.

———. 1910. *Das Nervensystem von Ascaris lumbricoides und megalocephala. Ein Versuch in den Aufbau eines einfachen Nervensystems einzudringen. III.*

(Festschrift zum sechzigsten Geburtstage Richard Hertwigs.).

Goodman, M. B., T. H. Lindsay, S. R. Lockery, and J. E. Richmond. 2012. 'Electrophysiological methods for Caenorhabditis elegans neurobiology', *Methods Cell Biol*, 107: 409-36.

Gotti, Cecilia, Michele Zoli, and Francesco Clementi. 2006. 'Brain nicotinic acetylcholine receptors: native subtypes and their relevance', *Trends in Pharmacological Sciences*, 27: 482-91.

Greenbaum, L., and B. Lerer. 2009. 'Differential contribution of genetic variation in multiple brain nicotinic cholinergic receptors to nicotine dependence: recent progress and emerging open questions', *Molecular Psychiatry*, 14: 912.

Greger, I. H., J. F. Watson, and S. G. Cull-Candy. 2017. 'Structural and Functional Architecture of AMPA-Type Glutamate Receptors and Their Auxiliary Proteins', *Neuron*, 94: 713-30.

Grove, David I. 1990. *A history of human helminthology.* (C.A.B. International: Wallingford, Oxon).

Guarrera, P. M. 1999. 'Traditional antihelmintic, antiparasitic and repellent uses of plants in Central Italy', *Journal of Ethnopharmacology*, 68: 183-92.

Guenther, E. 1948. *The essential oils. Vol. I. D.* (David van Nostrand Co. Inc, New York.).

Gurnett, C. A., and K. P. Campbell. 1996. 'Transmembrane Auxiliary Subunits of Voltage-dependent Ion Channels', *Journal of Biological Chemistry*, 271: 27975-78.

Gutierrez, Y. 2011. 'CHAPTER 112 - Other Tissue Nematode Infections.' in Richard L. Guerrant, David H. Walker and Peter F. Weller (eds.), *Tropical Infectious Diseases: Principles, Pathogens and Practice (Third Edition)* (W.B. Saunders: Edinburgh).

Halevi, S., J. P. McKay, M. Palfreyman, L. Yassin, M. Eshel, E. M. Jorgensen, and M. Treinin. 2002. 'The C.elegans ric-3 gene is required for maturation of nicotinic acetylcholine receptorss', *The EMBO Journal*, 21: 1012.

Hall, D. H., and E. M. Hedgecock. 1991. 'Kinesin-related gene unc-104 is required for axonal transport of synaptic vesicles in C. elegans', *Cell*, 65: 837-47.

Hall, D.H., and Z.F. Altun. 2008. 'Alimentary System, Pharynx.' in, *C. elegans Atlas* (CSHL Press).

Hamdan, F. F., M. D. Ungrin, M. Abramovitz, and P. Ribeiro. 1999. 'Characterization of a novel serotonin receptor from Caenorhabditis elegans: cloning and expression of two splice variants', *J Neurochem*, 72: 1372-83.

Harris, J. E., and H. D. Crofton. 1957a. 'Structure and Function in the Nematodes: Internal Pressure and Cuticular Structure in Ascaris', *Journal of Experimental Biology*, 34: 116.

———. 1957b. 'Structure and Function in the Nematodes: Internal Pressure and Cuticular Structure in Ascaris', *Journal of Experimental Biology*, 34: 116.

Haugstetter, J., T. Blicher, and L. Ellgaard. 2005. 'Identification and characterization of a novel thioredoxin-related transmembrane protein of the endoplasmic reticulum', *J Biol Chem*, 280: 8371-80.

Hernando, Guillermina, Ignacio Bergé, Diego Rayes, and Cecilia Bouzat. 2012. 'Contribution of subunits to *Caenorhabditis elegans* levamisole-sensitive nicotinic receptor function', *Molecular Pharmacology*, 82: 550-60.

Hoagland, K. E., and G. A. Schad. 1978. 'Necator americanus and Ancylostoma duodenale: life history parameters and epidemiological implications of two sympatric hookworms of humans', *Exp Parasitol*, 44: 36-49.

Hobson, R. J., J. Geng, A. D. Gray, and R. W. Komuniecki. 2003. 'SER-7b, a constitutively active Galphas coupled 5-HT7-like receptor expressed in the Caenorhabditis elegans M4 pharyngeal motorneuron', *J Neurochem*, 87: 22-9.

Hobson, R. J., V. M. Hapiak, H. Xiao, K. L. Buehrer, P. R. Komuniecki, and R. W. Komuniecki. 2006. 'SER-7, a Caenorhabditis elegans 5-HT7-like receptor, is essential for the 5-HT stimulation of pharyngeal pumping and egg laying', *Genetics*, 172: 159-69.

Hoeppli, R. 1956. 'The knowledge of parasites and parasitic infections from ancient times to the 17th century', *Experimental Parasitology*, 5: 398-419.

———. 1959. *Parasites and Parasitic Infections in Early Medicine and Science.* (University of Malaya Press, Singapore: Singapore).

Höld, K. M., N. S. Sirisoma, T. Ikeda, T. Narahashi, and J. E. Casida. 2000. 'α-Thujone (the active component of absinthe): γ-Aminobutyric acid type A receptor modulation and metabolic detoxification', *Proceedings of the National Academy of Sciences*, 97: 3826.

Holden-Dye, L., M. Joyner, V. O'Connor, and R. J. Walker. 2013. 'Nicotinic acetylcholine receptors: A comparison of the nAChRs of Caenorhabditis elegans and parasitic nematodes', *Parasitology International*, 62: 606-15.

Holden-Dye, L., and R. J. Walker. 2014. 'Anthelmintic drugs and nematicides: studies in Caenorhabditis elegans', *WormBook*: 1-29.

Horne, P. D. 1985. 'A review of the evidence of human endoparasitism in the pre-Columbian new world through the study of coprolites', *Journal of Archaeological Science*, 12: 299-310.

Horvitz, H. R., M. Chalfie, C. Trent, J. E. Sulston, and P. D. Evans. 1982. 'Serotonin and octopamine in the nematode Caenorhabditis elegans', *Science*, 216: 1012-4.

Hotez, P. 2008. 'Hookworm and poverty', *Ann N Y Acad Sci*, 1136: 38-44.

Hotez, P. J., P. J. Brindley, J. M. Bethony, C. H. King, E. J. Pearce, and J. Jacobson. 2008. 'Helminth infections: the great neglected tropical diseases', *The Journal of Clinical Investigation*, 118: 1311-21.

Hotez, P. J., S. Brooker, J. M. Bethony, M. E. Bottazzi, A. Loukas, and S. Xiao. 2004. 'Hookworm infection', *N Engl J Med*, 351: 799-807.

Hotez, Peter J., David H. Molyneux, Alan Fenwick, Jacob Kumaresan, Sonia Ehrlich Sachs, Jeffrey D. Sachs, and Lorenzo Savioli. 2007. 'Control of Neglected Tropical Diseases', *New England Journal of Medicine*, 357: 1018-27.

Hotez, Peter J., David H. Molyneux, Alan Fenwick, Eric Ottesen, Sonia Ehrlich Sachs, and Jeffrey D. Sachs. 2006. 'Incorporating a Rapid-Impact Package for Neglected Tropical Diseases with Programs for HIV/AIDS, Tuberculosis, and Malaria', *PLOS Medicine*, 3: e102.

Howe, J. R. 2015. 'Modulation of non-NMDA receptor gating by auxiliary subunits', *The Journal of Physiology*, 593: 61-72.

Hsü, H. F., Z. . 1929. 'Zellforsch. ', *Mikroskop. Anat.*, 9: 313.

Huang, K. C. 1998. *The pharmacology of chinese herbs.* (CRC Press, Inc., Boca Raton, Florida.).

Husson, S. J., A. Gottschalk, and A. M. Leifer. 2013. 'Optogenetic manipulation of neural activity in C. elegans: from synapse to circuits and behaviour', *Biol Cell*, 105: 235-50.

Isom, L. L., K. S. De Jongh, and W. A. Catterall. 1994. 'Auxiliary subunits of voltage-gated ion channels', *Neuron*, 12: 1183.

Itier, Valérie, and Daniel Bertrand. 2002. 'Mutations of the neuronal nicotinic acetylcholine receptors and their association with ADNFLE', *Neurophysiologie Clinique/Clinical Neurophysiology*, 32: 99-107.

Jaradat, N., L. Adwan, S. K'Aibni, N. Shraim, and A. N. Zaid. 2016. 'Chemical composition, anthelmintic, antibacterial and antioxidant effects of Thymus bovei essential oil', *BMC Complement Altern Med*, 16: 418.

Jarrett, E. E. E., and H. R. P. Miller. 1982. 'Production and Activities of IgE in Helminth Infection', *Chemical Immunology and Allergy*: 178-233.

John, White. 1988. '4 The Anatomy', *Cold Spring Harbor Monograph Archive; Volume 17 (1988): The Nematode Caenorhabditis elegans*.

Jolly, J., and C. G. Kashikar. 1951. *Indian medicine. Translated from German and supplemented with notes by C. G. Kashikar* (Poona [C. G. Kashikar]).

Jones, A. K., S. D. Buckingham, and D. B. Sattelle. 2005. 'Chemistry-to-gene screens in Caenorhabditis elegans', *Nat Rev Drug Discov*, 4: 321-30.

Jones, A. K., P. Davis, J. Hodgkin, and D. B. Sattelle. 2007. 'The nicotinic acetylcholine receptor gene family of the nematode Caenorhabditis elegans: an update on nomenclature', *Invertebrate neuroscience : IN*, 7: 129-31.

Jones, A. K., and D. B. Sattelle. 2004. 'Functional genomics of the nicotinic acetylcholine receptor gene family of the nematode, Caenorhabditis elegans', *BioEssays*, 26: 39-49.

Jones, K. E., N. G. Patel, M. A. Levy, A. Storeygard, D. Balk, J. L. Gittleman, and P. Daszak. 2008. 'Global trends in emerging infectious diseases', *Nature*, 451: 990.

Jones, W. H., and E. T. Whithington. 1948-1953. *Works of Hippocrates* (Loeb Classical Library, Heinemann, London, United Kingdom).

Jourdan, Peter Mark, Poppy H. L. Lamberton, Alan Fenwick, and David G. Addiss. 2018. 'Soil-transmitted helminth infections', *The Lancet*, 391: 252-65.

Kaletta, T., and M. O. Hengartner. 2006. 'Finding function in novel targets: C. elegans as a model organism', *Nat Rev Drug Discov*, 5: 387-98.

Kaminsky, R. , and L. Rufener. 2012. 'Monepantel: From Discovery to Mode of Action.' in C. R. Caffrey (ed.), *Parasitic Helminths: Targets, Screens, Drugs and Vaccines.* (Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, Germany.).

Kaminsky, R., P. Ducray, M. Jung, R. Clover, L. Rufener, J. Bouvier, S. S. Weber, A. Wenger, S. Wieland-Berghausen, T. Goebel, N. Gauvry, F. Pautrat, T. Skripsky, O. Froelich, C. Komoin-Oka, B. Westlund, A. Sluder, and P. Maser. 2008. 'A new class of anthelmintics effective against drug-resistant nematodes', *Nature*, 452: 176-80.

Kao, P. N., A. J. Dwork, R. R. Kaldany, M. L. Silver, J. Wideman, S. Stein, and A. Karlin. 1984. 'Identification of the alpha subunit half-cystine specifically labeled by an affinity reagent for the acetylcholine receptor binding site', *Journal of Biological Chemistry*, 259: 11662-65.

Kaplan, R. M., B. E. Storey, A. N. Vidyashankar, B. W. Bissinger, S. M. Mitchell, S. B. Howell, M. E. Mason, M. D. Lee, A. A. Pedroso, A. Akashe, and D. J. Skrypec. 2014. 'Antiparasitic efficacy of a novel plant-based functional food using an Ascaris suum model in pigs', *Acta Tropica*, 139: 15-22.

Kaplan, Ray M. 2004. 'Drug resistance in nematodes of veterinary importance: a status report', *Trends in Parasitology*, 20: 477-81.

Kapoor, L. D. 1990. *Handbook of ayurvedic medicinal plants.* (CRC Press, Inc., Boca Raton, Florida.).

Karlin, A. 2002. 'Emerging structure of the Nicotinic Acetylcholine receptors', *Nature Reviews Neuroscience*, 3: 102.

Katiki, L. M., A. C. Chagas, H. R. Bizzo, J. F. Ferreira, and A. F. Amarante. 2011. 'Anthelmintic activity of Cymbopogon martinii, Cymbopogon schoenanthus and Mentha piperita essential oils evaluated in four different in vitro tests', *Vet Parasitol*, 183: 103-8.

Katiki, L. M., J. F. Ferreira, A. M. Zajac, C. Masler, D. S. Lindsay, A. C. Chagas, and A. F. Amarante. 2011. 'Caenorhabditis elegans as a model to screen plant extracts and compounds as natural anthelmintics for veterinary use', *Vet Parasitol*, 182: 264-8.

Keane, J., and L. Avery. 2003. 'Mechanosensory inputs influence Caenorhabditis elegans pharyngeal activity via ivermectin sensitivity genes', *Genetics*, 164: 153-62.

Keiser, J., and J. Utzinger. 2008. 'Efficacy of current drugs against soil-transmitted helminth infections: systematic review and meta-analysis', *Jama*, 299: 1937-48.

Kerr, R. A. 2006. 'Imaging the activity of neurons and muscles', *WormBook*: 1-13.

Ki, H C., J. H. Bae, and D. H. Shin. 2013. 'Historical Study on Factors Inducing Soil-Transmitted Helminth Infection among People of Old Seoul City during Joseon Dynasty', *Korean J Med Hist*, 22: 89-132.

Kim, J., S. M. Seo, S. G. Lee, S. C. Shin, and I. K. Park. 2008. 'Nematicidal activity of plant essential oils and components from coriander (Coriandrum sativum), Oriental sweetgum (Liquidambar orientalis), and valerian (Valeriana wallichii) essential oils against pine wood nematode (Bursaphelenchus xylophilus)', *J Agric Food Chem*, 56: 7316-20.

King, Charles H. 2010. 'Parasites and poverty: The case of schistosomiasis', *Acta Tropica*, 113: 95-104.

Knopp, S., A. F. Mgeni, I. S. Khamis, P. Steinmann, J. R. Stothard, D. Rollinson, H. Marti, and J. Utzinger. 2008. 'Diagnosis of Soil-Transmitted Helminths in the Era of Preventive Chemotherapy: Effect of Multiple Stool Sampling and Use of Different Diagnostic Techniques', *PLOS Neglected Tropical Diseases*, 2: e331.

Kojima, S. 1999. '[Wakana disease]', *Ryoikibetsu Shokogun Shirizu*: 437-8.

Krepel, H. P., and A. M. Polderman. 1992. 'Egg production of Oesophagostomum bifurcum, a locally common parasite of humans in Togo', *Am J Trop Med Hyg*, 46: 469-72.

Kuryatov, Alexandre, Jennifer Onksen, and Jon Lindstrom. 2008. 'Roles of Accessory Subunits in α4β2* Nicotinic Receptors', *Molecular Pharmacology*, 74: 132.

Lamitina, T. 2006. 'Functional genomic approaches in C. elegans', *Methods Mol Biol*, 351: 127-38.

Lamson, P. D., and C. B. Ward. 1932. 'The chemotherapy of helminth infestations', *J. Parasitol.*, 18: 173-99.

Landmann, J. K., and P. Prociv. 2003. 'Experimental human infection with the dog hookworm, Ancylostoma caninum', *Med J Aust*, 178: 69-71.

Langley, J. N. 1901. 'Observations on the physiological action of extracts of the supra-renal bodies', *The Journal of Physiology*, 27: 237-56.

———. 1905. 'On the reaction of cells and of nerve-endings to certain poisons, chiefly as regards the reaction of striated muscle to nicotine and to curari', *The Journal of Physiology*, 33: 374-413.

Laughton, D. L., G. G. Lunt, and A. J. Wolstenholme. 1997. 'Alternative splicing of a Caenorhabditis elegans gene produces two novel inhibitory amino acid receptor subunits with identical ligand binding domains but different ion channels', *Gene*, 201: 119-25.

Le Novère, N., and J. P. Changeux. 1995. 'Molecular evolution of the nicotinic acetylcholine receptor: an example of multigene family in excitable cells', *J Mol Evol*, 40: 155-72.

Le Novère, N., P.-J. Corringer, and J.-P. Changeux. 1999. 'Improved Secondary Structure Predictions for a Nicotinic Receptor Subunit: Incorporation of Solvent Accessibility and Experimental Data into a Two-Dimensional Representation', *Biophysical Journal*, 76: 2329-45.

———. 2002. 'The diversity of subunit composition in nAChRs: Evolutionary origins, physiologic and pharmacologic consequences', *Journal of Neurobiology*, 53: 447-56.

Lee, D. L. 1965. *The physiology of nematodes* (San Francisco, W. H. Freeman: San Francisco).

Lee, D. L., and H. J. Atkinson. 1976. 'Neuro-muscular Physiology.' in D. L. Lee and H. J. Atkinson (eds.), *Physiology of Nematodes* (Macmillan Education UK: London).

Lee, R. Y., E. R. Sawin, M. Chalfie, H. R. Horvitz, and L. Avery. 1999. 'EAT-4, a homolog of a mammalian sodium-dependent inorganic phosphate cotransporter, is necessary for glutamatergic neurotransmission in caenorhabditis elegans', *J Neurosci*, 19: 159-67.

Lei, H., Y. Wang, F. Liang, W. Su, Y. Feng, X. Guo, and N. Wang. 2010. 'Composition and variability of essential oils of Platycladus orientalis growing in China', *Biochemical Systematics and Ecology*, 38: 1000-06.

Lei, J., M. Leser, and E. Enan. 2010. 'Nematicidal activity of two monoterpenoids and SER-2 tyramine receptor of Caenorhabditis elegans', *Biochem Pharmacol*, 79: 1062-71.

Leles, Daniela, Scott L. Gardner, Karl Reinhard, Alena Iñiguez, and Adauto Araujo. 2012. 'Are Ascaris lumbricoides and Ascaris suum a single species?', *Parasites & Vectors*, 5: 42.

Leonard, S., and D. Bertrand. 2001. 'Neuronal nicotinic receptors: from structure to function', *Nicotine & Tobacco Research*, 3: 203-23.

Leung, M. C., P. L. Williams, A. Benedetto, C. Au, K. J. Helmcke, M. Aschner, and J. N. Meyer. 2008. 'Caenorhabditis elegans: an emerging model in biomedical and environmental toxicology', *Toxicol Sci*, 106: 5-28.

Levin, E. D., and Barbara B. Simon. 1998. 'Nicotinic acetylcholine involvement in cognitive function in animals', *Psychopharmacology*, 138: 217-30.

Lewis, J. A., C. H. Wu, H. Berg, and J. H. Levine. 1980. 'The genetics of levamisole resistance in the nematode Caenorhabditis elegans', *Genetics*, 95: 905-28.

Li, Ming D., Zhongli Yang, Huazhang Guo, and Bhaghai Dash. 2016. 'Evolutionary Relationship of Nicotinic Acetylcholine Receptor Subunits in Both Vertebrate and Invertebrate Species.' in Ming D. Li (ed.), *Nicotinic Acetylcholine Receptor Technologies* (Springer New York: New York, NY).

Li, Zhaoyu, Jie Liu, Maohua Zheng, and X. Z. Shawn Xu. 2014. 'Encoding of Both Analog- and Digital-like Behavioral Outputs by One C. elegans Interneuron', *Cell*, 159: 751-65.

Lindquist, H. D. Alan, and John H. Cross. 2017. '195 - Helminths.' in Jonathan Cohen, William G. Powderly and Steven M. Opal (eds.), *Infectious Diseases (Fourth Edition)* (Elsevier).

Lindstrom, J. M. 2003. 'Nicotinic Acetylcholine Receptors of Muscles and Nerves', *Annals of the New York Academy of Sciences*, 998: 41-52.

Loukas, A., P. J. Hotez, D. Diemert, M. Yazdanbakhsh, J. S. McCarthy, R. Correa-Oliveira, J. Croese, and J. M. Bethony. 2016. 'Hookworm infection', *Nature Reviews Disease Primers*, 2: 16088.

Ma, Liang, Yudan Zhao, Yuchen Chen, Biao Cheng, Anlin Peng, and Kun Huang. 2018. 'Caenorhabditis elegans as a model system for target identification and drug screening against neurodegenerative diseases', *European Journal of Pharmacology*, 819: 169-80.

Macedo, I. T., C. M. Bevilaqua, L. M. de Oliveira, A. L. Camurca-Vasconcelos, S. Vieira Lda, F. R. Oliveira, E. M. Queiroz-Junior, B. G. Portela, R. S. Barros, and A. C. Chagas. 2009. 'Ovicidal and larvicidal activity in vitro of Eucalyptus globulus essential oils on Haemonchus contortus', *Rev Bras Parasitol Vet*, 18: 62-6.

Macklin, K. D., A. D. Maus, E. F. Pereira, E. X. Albuquerque, and B. M. Conti-Fine. 1998. 'Human vascular endothelial cells express functional nicotinic acetylcholine receptors', *J Pharmacol Exp Ther*, 287: 435-9.

Maher, M. P., J. A. Matta, S. Gu, M. Seierstad, and D. S. Bredt. 2017. 'Getting a Handle on Neuropharmacology by Targeting Receptor-Associated Proteins', *Neuron*, 96: 989-1001.

Mansvelder, Huibert D., and Daniel S. McGehee. 2002. 'Cellular and synaptic mechanisms of nicotine addiction', *Journal of Neurobiology*, 53: 606-17.

Mapes, C. J. 1965. 'Structure and function in the nematode pharynx: I. The structure of the pharynges of Ascaris lumbricoides, Oxyuris equi, Aplectana brevicaudata and Panagrellus silusiae', *Parasitology*, 55: 269-84.

———. 1966. 'Structure and function in the nematode pharynx. III. The pharyngeal pump of Ascaris lumbricoides', *Parasitology*, 56: 137-49.

Martin, R. J. 1993. 'Neuromuscular transmission in nematode parasites and antinematodal drug action', *Pharmacology & Therapeutics*, 58: 13-50.

Martin, R. J., A. J. Pennington, A. H. Duittoz, S. Robertson, and J. R. Kusel. 1991. 'The physiology and pharmacology of neuromuscular transmission in the nematode parasite, Ascaris suum', *Parasitology*, 102 Suppl: S41-58.

Martin, Richard J., Cheryl L. Clark, Sasa M. Trailovic, and Alan P. Robertson. 2004. 'Oxantel is an N-type (methyridine and nicotine) agonist not an L-type (levamisole and pyrantel) agonist: classification of cholinergic anthelmintics in Ascaris', *International Journal for Parasitology*, 34: 1083-90.

Martindale, R., and R. A. J. Lester. 2014. 'On the Discovery of the Nicotinic Acetylcholine Receptor Channel.' in Robin A. J. Lester (ed.), *Nicotinic Receptors* (Springer New York: New York, NY).

Maupas, E. 1900. 'Modes et formes de reproduction des nematodes.', *Archives de Zoologie Experimentale et Generale.*, 8: 463-624.

McKay, J. P., D. M. Raizen, A. Gottschalk, W. R. Schafer, and L. Avery. 2004. 'eat-2 and eat-18 are required for nicotinic neurotransmission in the Caenorhabditis elegans pharynx', *Genetics*, 166: 161-9.

Miledi, R., and L. T. Potter. 1971. 'Acetylcholine receptors in muscle fibres', *Nature*, 233: 599-603.

Millar, N. S. 2008. 'RIC-3: a nicotinic acetylcholine receptor chaperone', *British Journal of Pharmacology*, 153 Suppl 1: S177-S83.

Millar, N. S., and I. Denholm. 2007. 'Nicotinic acetylcholine receptors: targets for commercially important insecticides', *Invertebrate Neuroscience*, 7: 53-66.

Millar, N. S., and C. Gotti. 2009. 'Diversity of vertebrate nicotinic acetylcholine receptors', *Neuropharmacology*, 56: 237-46.

Missias, Andrea C., Gerald C. Chu, Barbara J. Klocke, Joshua R. Sanes, and John P. Merlie. 1996. 'Maturation of the Acetylcholine Receptor in Skeletal Muscle: Regulation of the AChR γ-to-ϵ Switch', *Developmental Biology*, 179: 223-38.

Miyazawa, A., Y. Fujiyoshi, M. Stowell, and N. Unwin. 1999. 'Nicotinic acetylcholine receptor at 4.6 Å resolution: transverse tunnels in the channel11Edited by J. Karn', *Journal of Molecular Biology*, 288: 765-86.

Miyazawa, M., H. Watanabe, and H. Kameoka. 1997. 'Inhibition of Acetylcholinesterase Activity by Monoterpenoids with a p-Menthane Skeleton', *Journal of Agricultural and Food Chemistry*, 45: 677-79.

Monassier, L. 2017. '[Claude Bernard and nicotinic receptors: from the neuromuscular junction to tobacco weaning]', *Biol Aujourdhui*, 211: 169-72.

Mongan, N. P., H. A. Baylis, C. Adcock, G. R. Smith, M. S. Sansom, and D. B. Sattelle. 1998. 'An extensive and diverse gene family of nicotinic acetylcholine receptor alpha subunits in Caenorhabditis elegans', *Receptors Channels*, 6: 213-28.

Mongan, N. P., A. K. Jones, G. R. Smith, M. S. P. Sansom, and D. B. Sattelle. 2002. 'Novel alpha7-like nicotinic acetylcholine receptor subunits in the nematode Caenorhabditis elegans', *Protein science : a publication of the Protein Society*, 11: 1162-71.

Nasiripourdori, Adak, Valérie Taly, Thomas Grutter, and Antoine Taly. 2011. 'From Toxins Targeting Ligand Gated Ion Channels to Therapeutic Molecules', *Toxins*, 3: 260-93.

Nayak, T. K., and A. Auerbach. 2013. 'Asymmetric transmitter binding sites of fetal muscle acetylcholine receptors shape their synaptic response', *Proceedings of the National Academy of Sciences of the United States of America*, 110: 13654-59.

Nejsum, P., M. Betson, R. P. Bendall, S. M. Thamsborg, and J. R. Stothard. 2012. 'Assessing the zoonotic potential of Ascaris suum and Trichuris suis: looking to the future from an analysis of the past', *Journal of Helminthology*, 86: 148-55.

Neveu, Cédric, Claude L. Charvet, Aymeric Fauvin, Jacques Cortet, Robin N. Beech, and Jacques Cabaret. 2010. 'Genetic diversity of levamisole receptor subunits in parasitic nematode species and abbreviated transcripts associated with resistance', *Pharmacogenetics and Genomics*, 20: 414-25.

Newman, D. J., and G. M. Cragg. 2016. 'Natural Products as Sources of New Drugs from 1981 to 2014', *J Nat Prod*, 79: 629-61.

Newman, D. J., G. M. Cragg, and K. M. Snader. 2000. 'The influence of natural products upon drug discovery', *Nat Prod Rep*, 17: 215-34.

Newman, Morgan, Doris Kretzschmar, Imran Khan, Mengqi Chen, Giuseppe Verdile, and Michael Lardelli. 2017. 'Chapter 40 - Animal Models of Alzheimer's Disease.' in P. Michael Conn (ed.), *Animal Models for the Study of Human Disease (Second Edition)* (Academic Press).

Ngui, R., Y. A. Lim, R. Traub, R. Mahmud, and M. S. Mistam. 2012. 'Epidemiological and genetic data supporting the transmission of Ancylostoma ceylanicum among human and domestic animals', *PLoS Negl Trop Dis*, 6: e1522.

Nikolay, B., S. J. Brooker, and R. L. Pullan. 2014. 'Sensitivity of diagnostic tests for human soil-transmitted helminth infections: a meta-analysis in the absence of a true gold standard', *International Journal for Parasitology*, 44: 765-74.

Ntalli, N. G., F. Ferrari, I. Giannakou, and U. Menkissoglu-Spiroudi. 2010. 'Phytochemistry and nematicidal activity of the essential oils from 8 Greek Lamiaceae aromatic plants and 13 terpene components', *Journal of Agricultural and Food Chemistry*, 58: 7856.

Nussbaum-Krammer, C. I., and R. I. Morimoto. 2014. 'Caenorhabditis elegans as a model system for studying non-cell-autonomous mechanisms in protein-misfolding diseases', *Dis Model Mech*, 7: 31-9.

O'Malley, H. A., and L. L. Isom. 2015. 'Sodium Channel β Subunits: Emerging Targets in Channelopathies', *Annual Review of Physiology*, 77: 481-504.

Okulewicz, A., A. Perec, and J. Hildebrand. 2005. 'Biodiversity of nematode fauna.', *Wiad Parazytol*, 51: 209-12.

Osten, P., and Y. Stern-Bach. 2006. 'Learning from stargazin: the mouse, the phenotype and the unexpected', *Current Opinion in Neurobiology*, 16: 275-80.

Panella, N. A., M. C. Dolan, J. J. Karchesy, Y. Xiong, J. Peralta-Cruz, M. Khasawneh, J. A. Montenieri, and G. O. Maupin. 2005. 'Use of novel compounds for pest control: insecticidal and acaricidal activity of essential oil components from heartwood of Alaska yellow cedar', *J Med Entomol*, 42: 352-8.

Park, Tae-Ju, Yong-Soo Park, Tae-Gyun Lee, Hyunjung Ha, and Kyong-Tai Kim. 2003. 'Inhibition of acetylcholine-mediated effects by borneol', *Biochemical Pharmacology*, 65: 83-90.

Paterson, David, and Agneta Nordberg. 2000. 'Neuronal nicotinic receptors in the human brain', *Progress in Neurobiology*, 61: 75-111.

Patrick, J., M. Ballivet, L. Boas, T. Claudio, J. Forrest, H. Ingraham, P. Mason, S. Stengelin, S. Ueno, and S. Heinemann. 1983. 'Molecular cloning of the acetylcholine receptor', *Cold Spring Harb Symp Quant Biol*, 48 Pt 1: 71-8.

Patrucco, R., R. Tello, and D. Bonavia. 1983. 'Parasitological Studies of Coprolites of Pre-Hispanic Peruvian Populations', *Current Anthropology*, 24: 393-94.

Patton, A., S. Knuth, B. Schaheen, H. Dang, I. Greenwald, and H. Fares. 2005. 'Endocytosis Function of a Ligand-Gated Ion Channel Homolog in Caenorhabditis elegans', *Current Biology*, 15: 1045-50.

Pedersen, Julia E., Christina A. Bergqvist, and Dan Larhammar. 2019. 'Evolution of vertebrate nicotinic acetylcholine receptors', *BMC evolutionary biology*, 19: 38-38.

Pemberton, D. J., C. J. Franks, R. J. Walker, and L. Holden-Dye. 2001. 'Characterization of glutamate-gated chloride channels in the pharynx of wild-type and mutant Caenorhabditis elegans delineates the role of the subunit GluCl-alpha2 in the function of the native receptor', *Mol Pharmacol*, 59: 1037-43.

Pennington, A. J., and R. J. Martin. 1990. 'A patch-clamp study of acetylcholine-activated ion channels in Ascaris suum muscle', *J Exp Biol*, 154: 201-21.

Phelan, P. 2005. 'Innexins: members of an evolutionarily conserved family of gap-junction proteins', *Biochim Biophys Acta*, 1711: 225-45.

Picciotto, M. R., and M. Zoli. 2002. 'Nicotinic receptors in aging and dementia', *Journal of Neurobiology*, 53: 641-55.

Poinar, George O. 2015. 'Chapter 14 - Phylum Nemata.' in James H. Thorp and D. Christopher Rogers (eds.), *Thorp and Covich's Freshwater Invertebrates (Fourth Edition)* (Academic Press: Boston).

Polderman, A. M., and J. Blotkamp. 1995. 'Oesophagostomum infections in humans', *Parasitol Today*, 11: 451-6.

Polderman, A. M., M. Eberhard, S. Baeta, Robin B. Gasser, L. van Lieshout, P. Magnussen, A. Olsen, N. Spannbrucker, J. Ziem, and J. Horton. 2010. 'Chapter 3 - The Rise and Fall of Human Oesophagostomiasis.' in, *Advances in Parasitology* (Academic Press).

Polderman, A. M., H. P. Krepel, S. Baeta, J. Blotkamp, and P. Gigase. 1991. 'Oesophagostomiasis common infection of man in northern Togo and Ghana', 4: 336-44.

Prichard, R. K., M. G. Basanez, B. A. Boatin, J. S. McCarthy, H. H. Garcia, G. J. Yang, B. Sripa, and S. Lustigman. 2012. 'A research agenda for helminth diseases of humans: intervention for control and elimination', *PLoS Negl Trop Dis*, 6: e1549.

Prociv, P., and J. Croese. 1996. 'Human enteric infection with Ancylostoma caninum: hookworms reappraised in the light of a "new" zoonosis', *Acta Trop*, 62: 23-44.

Pullan, Rachel L., Jennifer L. Smith, Rashmi Jasrasaria, and Simon J. Brooker. 2014. 'Global numbers of infection and disease burden of soil transmitted helminth infections in 2010', *Parasites & Vectors*, 7: 37.

Qian, Hai, Richard J. Martin, and Alan P. Robertson. 2006. 'Pharmacology of N-, L-, and B-subtypes of nematode nAChR resolved at the single-channel level in Ascaris suum', *The FASEB Journal*, 20: 2606-08.

Raizen, D. M., and L. Avery. 1994. 'Electrical activity and behavior in the pharynx of Caenorhabditis elegans', *Neuron*, 12: 483-95.

Raizen, D. M., R. Y. Lee, and L. Avery. 1995. 'Interacting genes required for pharyngeal excitation by motor neuron MC in Caenorhabditis elegans', *Genetics*, 141: 1365-82.

Ranganathan, R., S. C. Cannon, and H. R. Horvitz. 2000. 'MOD-1 is a serotonin-gated chloride channel that modulates locomotory behaviour in C. elegans', *Nature*, 408: 470-5.

Rankin, C. H. 2002. 'From gene to identified neuron to behaviour in Caenorhabditis elegans', *Nat Rev Genet*, 3: 622-30.

Raymond, V., N. P. Mongan, and D. B. Sattelle. 2000. 'Anthelmintic actions on homomer-forming nicotinic acetylcholine receptor subunits: chicken α7 and ACR-16 from the nematode Caenorhabditis elegans', *Neuroscience*, 101: 785-91.

Reger, J. F. 1966. 'The fine structure of fibrillar components and plasma membrane contacts in esophageal myoepithelium of Ascaris lumbricoides (var. suum)', *Journal of Ultrastructure Research*, 14: 602-17.

Rex, E., S. C. Molitor, V. Hapiak, H. Xiao, M. Henderson, and R. Komuniecki. 2004. 'Tyramine receptor (SER-2) isoforms are involved in the regulation of pharyngeal pumping and foraging behavior in Caenorhabditis elegans', *Journal of Neurochemistry*, 91: 1104-15.

Richmond, J. E., and E. M. Jorgensen. 1999. 'One GABA and two acetylcholine receptors function at the C. elegans neuromuscular junction', *Nature Neuroscience*, 2: 791-97.

Riddle, D. L., T. Blumenthal, B. J. Meyer, and J. R. Priess. 1997. *C. elegans II* (
Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Rieckher, Matthias, Nikos Kourtis, Angela Pasparaki, and Nektarios Tavernarakis. 2009. 'Transgenesis in Caenorhabditis elegans.' in Elizabeth J. Cartwright (ed.), *Transgenesis Techniques: Principles and Protocols* (Humana Press: Totowa, NJ).

Roberts, A., and C. Kemp. 2001. 'Ascariasis', *Journal of the American Academy of Nurse Practitioners*, 13: 55.

Robertson, A. P., H. E. Bjorn, and R. J. Martin. 1999. 'Resistance to levamisole resolved at the single-channel level.', *Faseb J.*, 13: 749-60.

Robertson, A. P., H. E. Bjørn, and R. J. Martin. 2000. 'Pyrantel resistance alters nematode nicotinic acetylcholine receptor single-channel properties', *European Journal of Pharmacology*, 394: 1-8.

Robertson, A. P., C. L. Clark, T. A. Burns, D. P. Thompson, T. G. Geary, S. M. Trailovic, and R. J. Martin. 2002. 'Paraherquamide and 2-Deoxy-paraherquamide Distinguish Cholinergic Receptor Subtypes in Ascaris Muscle', *Journal of Pharmacology and Experimental Therapeutics*, 302: 853.

Robertson, S. J., and R. J. Martin. 1993. 'Levamisole-activated single-channel currents from muscle of the nematode parasite Ascaris suum', *British Journal of Pharmacology*, 108: 170-78.

Robertson, S. J., A. J. Pennington, A. Mark Evans, and R. J. Martin. 1994. 'The action of pyrantel as an agonist and an open channel blocker at acetylcholine receptors in isolated Ascaris suum muscle vesicles', *European Journal of Pharmacology*, 271: 273-82.

Rogers, C. M., C. J. Franks, R. J. Walker, J. F. Burke, and L. Holden-Dye. 2001. 'Regulation of the pharynx of Caenorhabditis elegans by 5-HT, octopamine, and FMRFamide-like neuropeptides', *Journal of Neurobiology*, 49: 235-44.

Rosenbluth, J. 1963. 'Fine structure of body muscle cells and neuromuscular junctions in Ascaris lumbricoides. ', *J. Cell. Biol.*, 19: 82A.

———. 1965a. 'Ultrastructural organization of obliquely striated muscle fibers in Ascaris lumbricoides', *The Journal of Cell Biology*, 25: 495-515.

———. 1965b. 'Ultrastructure of somatic muscle cells in Ascaris lumbricoides. II. Intermuscular junctions, neuromuscular junctions, and glycogen stores', *The Journal of Cell Biology*, 26: 579-91.

Rufener, L., N. Bedoni, R. Baur, S. Rey, D. A. Glauser, J. Bouvier, R. Beech, E. Sigel, and A. Puoti. 2013. 'acr-23 Encodes a monepantel-sensitive channel in Caenorhabditis elegans', *PLoS Pathog*, 9: e1003524.

Sacko, M., D. De Clercq, J. M. Behnke, F. S. Gilbert, P. Dorny, and J. Vercruysse. 1999. 'Comparison of the efficacy of mebendazole, albendazole and pyrantel in treatment of human hookworm infections in the southern region of Mali, West Africa', *Trans R Soc Trop Med Hyg*, 93: 195-203.

Samuelsson, G. 2004. *Drugs of Natural Origin: A Textbook of Pharmacognosy, 5th Edn.* (Swedish Pharmaceutical Press.: Stockholm).

Sanyal, S., R. F. Wintle, K. S. Kindt, W. M. Nuttley, R. Arvan, P. Fitzmaurice, E. Bigras, D. C. Merz, T. E. Hébert, D. van der Kooy, W. R. Schafer, J. G. Culotti, and H. H. M. Van Tol. 2004. 'Dopamine modulates the plasticity of mechanosensory responses in Caenorhabditis elegans', *The EMBO Journal*, 23: 473-82.

Sauders, J. R., and A. H. Burr. 1978. 'The pumping mechanism of the nematode esophagus', *Biophysical Journal*, 22: 349-72.

Saur, T., S. E. DeMarco, A. Ortiz, G. R. Sliwoski, L. Hao, X. Wang, B. M. Cohen, and E. A. Buttner. 2013. 'A genome-wide RNAi screen in Caenorhabditis elegans identifies the nicotinic acetylcholine receptor subunit ACR-7 as an antipsychotic drug target', *PLoS Genet*, 9: e1003313.

Sawin, E. R., R. Ranganathan, and H. R. Horvitz. 2000. 'C. elegans locomotory rate is modulated by the environment through a dopaminergic pathway and by experience through a serotonergic pathway', *Neuron*, 26: 619-31.

Schneider, A. . 1866. 'Monographie der Nematoden': Berlin.

Seguela, P., J. Wadiche, K. Dineley-Miller, J. A. Dani, and J. W. Patrick. 1993. 'Molecular cloning, functional properties, and distribution of rat brain alpha 7: a nicotinic cation channel highly permeable to calcium', *The Journal of Neuroscience*, 13: 596.

Seymour, M. K., K. A. Wright, and C. C. Doncaster. 1983. 'The action of the anterior feeding apparatus of Caenorhabditis elegans (Nematoda: Rhabditida)', *Journal of Zoology*, 201: 527-39.

Shah, Jamil, and Abul Shahidullah. 2018. 'Ascaris lumbricoides: A Startling Discovery during Screening Colonoscopy', *Case reports in gastroenterology*, 12: 224-29.

Sine, S. M., and A. G. Engel. 2006. 'Recent advances in Cys-loop receptor structure and function', *Nature*, 440: 448-55.

Sinniah, B. 1982. 'Daily egg production of Ascaris lumbricoides: the distribution of eggs in the faeces and the variability of egg counts', *Parasitology*, 84: 167-75.

Smit, R. B., R. Schnabel, and J. Gaudet. 2008. 'The HLH-6 transcription factor regulates C. elegans pharyngeal gland development and function', *PLoS Genet*, 4: e1000222.

Steinlein, Ortrud K., John C. Mulley, Peter Propping, Robyn H. Wallace, Hilary A. Phillips, Grant R. Sutherland, Ingrid E. Scheffer, and Samuel F. Berkovic. 1995. 'A missense mutation in the neuronal nicotinic acetylcholine receptor α4 subunit is associated with autosomal dominant nocturnal frontal lobe epilepsy', *Nature Genetics*, 11: 201-03.

Stiernagle, T. 2006. 'Maintenance of C. elegans', *WormBook*: 1-11.

Stolk, Wilma A., Margarete C. Kulik, Epke A. le Rutte, Julie Jacobson, Jan Hendrik Richardus, Sake J. de Vlas, and Tanja A. J. Houweling. 2016. 'Between-Country Inequalities in the Neglected Tropical Disease Burden in 1990 and 2010, with Projections for 2020', *PLOS Neglected Tropical Diseases*, 10: e0004560.

Stoltzfus, R. J., H. M. Chwaya, J. M. Tielsch, K. J. Schulze, M. Albonico, and L. Savioli. 1997. 'Epidemiology of iron deficiency anemia in Zanzibari schoolchildren: the importance of hookworms', *Am J Clin Nutr*, 65: 153-9.

Stoltzfus, R. J., M. L. Dreyfuss, H. M. Chwaya, and M. Albonico. 1997. 'Hookworm Control as a Strategy to Prevent Iron Deficiency', *Nutrition Reviews*, 55: 223-32.

Storey, P. A., G. Faile, E. Hewitt, L. Yelifari, A. M. Polderman, and P. Magnussen. 2000. 'Clinical epidemiology and classification of human oesophagostomiasis', *Transactions of The Royal Society of Tropical Medicine and Hygiene*, 94: 177-82.

Storey, P. A., N. Spannbrucker, E. A. Agongo, L. van Lieshout, J. P. Zeim, P. Magnussen, A. M. Polderman, and E. Doehring. 2002. 'Intraobserver and interobserver variation of ultrasound diagnosis of Oesophagostomum bifurcum colon lesions', *Am J Trop Med Hyg*, 67: 680-3.

Storey, P. A., N. R. Steenhard, L. Van Lieshout, S. Anemana, P. Magnussen, and A. M. Polderman. 2001. 'Natural progression of Oesophagostomum bifurcum pathology and infection in a rural community of northern Ghana', *Trans R Soc Trop Med Hyg*, 95: 295-9.

Stretton, A., J. Donmoyer, R. Davis, J. Meade, C. Cowden, and P. Sithigorngul. 1992. 'Motor Behavior and Motor Nervous System Function in the Nematode Ascaris suum', *The Journal of Parasitology*, 78: 206-14.

Stretton, A. O. 1976. 'Anatomy and development of the somatic musculature of the nematode Ascaris', *Journal of Experimental Biology*, 64: 773.

Stretton, A. O., R. M. Fishpool, E. Southgate, J. E. Donmoyer, J. P. Walrond, J. E. Moses, and I. S. Kass. 1978. 'Structure and physiological activity of the motoneurons of the nematode Ascaris', *Proceedings of the National Academy of Sciences*, 75: 3493.

Sulston, J., M. Dew, and S. Brenner. 1975. 'Dopaminergic neurons in the nematode Caenorhabditis elegans', *Journal of Comparative Neurology*, 163: 215-26.

Sulston, J. E., E. Schierenberg, J. G. White, and J. N. Thomson. 1983. 'The embryonic cell lineage of the nematode Caenorhabditis elegans', *Dev Biol*, 100: 64-119.

Sumikawa, K., M. Houghton, J. C. Smith, L. Bell, B. M. Richards, and E. A. Barnard. 1982. 'The molecular cloning and characterisation of cDNA coding for the α subunit of the acetyl-choline receptor', *Nucleic Acids Research*, 10: 5809-22.

Sze, J. Y., M. Victor, C. Loer, Y. Shi, and G. Ruvkun. 2000. 'Food and metabolic signalling defects in a Caenorhabditis elegans serotonin-synthesis mutant', *Nature*, 403: 560-4.

Taman, A., and M. Azab. 2014. 'Present-day anthelmintics and perspectives on future new targets', *Parasitol Res*, 113: 2425-33.

Teschendorf, D., and C. D. Link. 2009. 'What have worm models told us about the mechanisms of neuronal dysfunction in human neurodegenerative diseases?', *Mol Neurodegener*, 4: 38.

Theis, Nina, and Manuel Lerdau. 2003. 'The Evolution of Function in Plant Secondary Metabolites', *International Journal of Plant Sciences*, 164: S93-S102.

Thompson, A. J., H. A. Lester, and S. C. R. Lummis. 2010. 'The structural basis of function in Cys-loop receptors', *Quarterly Reviews of Biophysics*, 43: 449-99.

Tietze, P. E., and P. H. Tietze. 1991. 'The roundworm, Ascaris lumbricoides', *Prim Care*, 18: 25-41.

Tomita, S. 2010. 'Regulation of ionotropic glutamate receptors by their auxiliary subunits', *Physiology (Bethesda, Md.)*, 25: 41-49.

Tong, F., and J. R. Coats. 2012. 'Quantitative structure-activity relationships of monoterpenoid binding activities to the housefly GABA receptor', *Pest Manag Sci*, 68: 1122-9.

Tong, F., A. D. Gross, M. C. Dolan, and J. R. Coats. 2013. 'The phenolic monoterpenoid carvacrol inhibits the binding of nicotine to the housefly nicotinic acetylcholine receptor', *Pest Manag Sci*, 69: 775-80.

Touroutine, D., R. M. Fox, S. E. Von Stetina, A. Burdina, D. M. Miller, and J. E. Richmond. 2005. 'acr-16 Encodes an Essential Subunit of the Levamisole-resistant Nicotinic Receptor at the Caenorhabditis elegans Neuromuscular Junction', *Journal of Biological Chemistry*, 280: 27013-21.

Towers, P. R., B. Edwards, J. E. Richmond, and D. B. Sattelle. 2005. 'The Caenorhabditis elegans lev-8 gene encodes a novel type of nicotinic acetylcholine receptor α subunit', *Journal of Neurochemistry*, 93: 1-9.

Trailović, S. M., D. S. Marjanovic, J. Nedeljkovic Trailovic, A. P. Robertson, and R. J. Martin. 2015. 'Interaction of carvacrol with the Ascaris suum nicotinic acetylcholine receptors and gamma-aminobutyric acid receptors, potential mechanism of antinematodal action', *Parasitol Res*, 114: 3059-68.

Traversa, D. 2012. 'Pet roundworms and hookworms: a continuing need for global worming', *Parasit Vectors*, 5: 91.

Treinin, M., B. Gillo, L. Liebman, and M. Chalfie. 1998. 'Two functionally dependent acetylcholine subunits are encoded in a single Caenorhabditis elegans operon', *Proceedings of the National Academy of Sciences*, 95: 15492.

Trojanowski, N. F. , O. Padovan-Merhar, D. M. Raizen, and C. Fang-Yen. 2014. 'Neural and genetic degeneracy underlies Caenorhabditis elegans feeding behavior', *Journal of Neurophysiology*, 112: 951-61.

Trojanowski, N. F. , D. M. Raizen, and C. Fang-Yen. 2016. 'Pharyngeal pumping in Caenorhabditis elegans depends on tonic and phasic signaling from the nervous system', *Scientific Reports*, 6: 22940.

Unwin, N. 2005. 'Refined Structure of the Nicotinic Acetylcholine Receptor at 4Å Resolution', *Journal of Molecular Biology*, 346: 967-89.

Vandenberghe, W., R. A. Nicoll, and D. S. Bredt. 2005. 'Stargazin is an AMPA receptor auxiliary subunit', *Proceedings of the National Academy of Sciences of the United States of America*, 102: 485-90.

Vassilatis, D. K., K. O. Elliston, P. S. Paress, M. Hamelin, J. P. Arena, J. M. Schaeffer, L. H.T. Van der Ploeg, and D. F. Cully. 1997. 'Evolutionary Relationship of the Ligand-Gated Ion Channels and the Avermectin-Sensitive, Glutamate-Gated Chloride Channels', *Journal of Molecular Evolution*, 44: 501-08.

Verma, S., S. S. Kashyap, A. P. Robertson, and R. J. Martin. 2017. 'Functional genomics in Brugia malayi reveal diverse muscle nAChRs and differences between cholinergic anthelmintics', *Proc Natl Acad Sci U S A*, 114: 5539-44.

Verweij, J. J., D. S. S. Pit, L. Van Lieshout, S. M. Baeta, G. D. Dery, R. B. Gasser, and A. M. Polderman. 2001. 'Determining the prevalence of Oesophagostomum bifurcum

and Necator americanus infections using specific PCR amplification of DNA from faecal samples', *Tropical Medicine & International Health*, 6: 726-31.

Vos, Theo, Amanuel Alemu Abajobir, Kalkidan Hassen Abate, Cristiana Abbafati, Kaja M. Abbas, Foad Abd-Allah, Rizwan Suliankatchi Abdulkader, Abdishakur M. Abdulle, Teshome Abuka Abebo, Semaw Ferede Abera, Victor Aboyans, Laith J. Abu-Raddad, Ilana N. Ackerman, Abdu Abdullahi Adamu, Olatunji Adetokunboh, Mohsen Afarideh, Ashkan Afshin, Sanjay Kumar Agarwal, Rakesh Aggarwal, Anurag Agrawal, Sutapa Agrawal, Hamid Ahmadieh, Muktar Beshir Ahmed, Miloud Taki Eddine Aichour, Amani Nidhal Aichour, Ibtihel Aichour, Sneha Aiyar, Rufus Olusola Akinyemi, Nadia Akseer, Faris Hasan Al Lami, Fares Alahdab, Ziyad Al-Aly, Khurshid Alam, Noore Alam, Tahiya Alam, Deena Alasfoor, Kefyalew Addis Alene, Raghib Ali, Reza Alizadeh-Navaei, Ala'a Alkerwi, François Alla, Peter Allebeck, Christine Allen, Fatma Al-Maskari, Rajaa Al-Raddadi, Ubai Alsharif, Shirina Alsowaidi, Khalid A. Altirkawi, Azmeraw T. Amare, Erfan Amini, Walid Ammar, Yaw Ampem Amoako, Hjalte H. Andersen, Carl Abelardo T. Antonio, Palwasha Anwari, Johan Ärnlöv, Al Artaman, Krishna Kumar Aryal, Hamid Asayesh, Solomon W. Asgedom, Reza Assadi, Tesfay Mehari Atey, Niguse Tadele Atnafu, Sachin R. Atre, Leticia Avila-Burgos, Euripide Frinel G. Arthur Avokphako, Ashish Awasthi, Umar Bacha, Alaa Badawi, Kalpana Balakrishnan, Amitava Banerjee, Marlena S. Bannick, Aleksandra Barac, Ryan M. Barber, Suzanne L. Barker-Collo, Till Bärnighausen, Simon Barquera, Lars Barregard, Lope H. Barrero, Sanjay Basu, Bob Battista, Katherine E. Battle, Bernhard T. Baune, Shahrzad Bazargan-Hejazi, Justin Beardsley, Neeraj Bedi, Ettore Beghi, Yannick Béjot, Bayu Begashaw Bekele, Michelle L. Bell, Derrick A. Bennett, Isabela M. Bensenor, Jennifer Benson, Adugnaw Berhane, Derbew Fikadu Berhe, Eduardo Bernabé, Balem Demtsu Betsu, Mircea Beuran, Addisu Shunu Beyene, Neeraj Bhala, Anil Bhansali, Samir Bhatt, Zulfiqar A. Bhutta, Sibhatu Biadgilign, Burcu Kucuk Bicer, Kelly Bienhoff, Boris Bikbov, Charles Birungi, Stan Biryukov, Donal Bisanzio, Habtamu Mellie Bizuayehu, Dube Jara Boneya, Soufiane Boufous, Rupert R. A. Bourne, Alexandra Brazinova, Traolach S. Brugha, Rachelle Buchbinder, Lemma Negesa Bulto Bulto, Blair R. Bumgarner, Zahid A. Butt, Lucero Cahuana-Hurtado, Ewan Cameron, Mate Car, Hélène Carabin, Jonathan R. Carapetis, Rosario Cárdenas, David O. Carpenter, Juan Jesus Carrero, Austin Carter, Felix Carvalho, Daniel C. Casey, Valeria Caso, Carlos A. Castañeda-Orjuela, Chris D. Castle, Ferrán Catalá-López, Hsing-Yi Chang, Jung-Chen Chang, Fiona J. Charlson, Honglei Chen, Mirriam Chibalabala, Chioma Ezinne Chibueze, Vesper Hichilombwe Chisumpa, Abdulaal A. Chitheer, Devasahayam Jesudas Christopher, Liliana G. Ciobanu, Massimo Cirillo, Danny Colombara, Cyrus Cooper, Paolo Angelo Cortesi, Michael H. Criqui, John A. Crump, Abel Fekadu Dadi, Koustuv Dalal, Lalit Dandona, Rakhi Dandona, José das Neves, Dragos V. Davitoiu, Barbora de Courten, Diego De De Leo, Barthelemy Kuate Defo, Louisa Degenhardt, Selina Deiparine, Robert P. Dellavalle, Kebede Deribe, Don C. Des Jarlais, Subhojit Dey, Samath D. Dharmaratne, Preet Kaur Dhillon, Daniel Dicker, Eric L. Ding, Shirin Djalalinia, Huyen Phuc Do, E. Ray Dorsey, Kadine Priscila Bender dos Santos, Dirk Douwes-Schultz, Kerrie E. Doyle, Tim R. Driscoll, Manisha Dubey, Bruce Bartholow Duncan, Ziad Ziad El-Khatib, Jerisha

Ellerstrand, Ahmadali Enayati, Aman Yesuf Endries, Sergey Petrovich Ermakov, Holly E. Erskine, Babak Eshrati, Sharareh Eskandarieh, Alireza Esteghamati, Kara Estep, Fanuel Belayneh Bekele Fanuel, Carla Sofia E. Sa Farinha, André Faro, Farshad Farzadfar, Mir Sohail Fazeli, Valery L. Feigin, Seyed-Mohammad Fereshtehnejad, João C. Fernandes, Alize J. Ferrari, Tesfaye Regassa Feyissa, Irina Filip, Florian Fischer, Christina Fitzmaurice, Abraham D. Flaxman, Luisa Sorio Flor, Nataliya Foigt, Kyle J. Foreman, Richard C. Franklin, Nancy Fullman, Thomas Fürst, Joao M. Furtado, Neal D. Futran, Emmanuela Gakidou, Morsaleh Ganji, Alberto L. Garcia-Basteiro, Teshome Gebre, Tsegaye Tewelde Gebrehiwot, Ayele Geleto, Bikila Lencha Gemechu, Hailay Abrha Gesesew, Peter W. Gething, Alireza Ghajar, Katherine B. Gibney, Paramjit Singh Gill, Richard F. Gillum, Ibrahim Abdelmageem Mohamed Ginawi, Ababi Zergaw Giref, Melkamu Dedefo Gishu, Giorgia Giussani, William W. Godwin, Audra L. Gold, Ellen M. Goldberg, Philimon N. Gona, Amador Goodridge, Sameer Vali Gopalani, Atsushi Goto, Alessandra Carvalho Goulart, Max Griswold, Harish Chander Gugnani, Rahul Gupta, Rajeev Gupta, Tanush Gupta, Vipin Gupta, Nima Hafezi-Nejad, Gessessew Bugssa Hailu, Alemayehu Desalegne Hailu, Randah Ribhi Hamadeh, Samer Hamidi, Alexis J. Handal, Graeme J. Hankey, Sarah Wulf Hanson, Yuantao Hao, Hilda L. Harb, Habtamu Abera Hareri, Josep Maria Haro, James Harvey, Mohammad Sadegh Hassanvand, Rasmus Havmoeller, Caitlin Hawley, Simon I. Hay, Roderick J. Hay, Nathaniel J. Henry, Ileana Beatriz Heredia-Pi, Julio Montañez Hernandez, Pouria Heydarpour, Hans W. Hoek, Howard J. Hoffman, Nobuyuki Horita, H. Dean Hosgood, Sorin Hostiuc, Peter J. Hotez, Damian G. Hoy, Aung Soe Htet, Guoqing Hu, Hsiang Huang, Chantal Huynh, Kim Moesgaard Iburg, Ehimario Uche Igumbor, Chad Ikeda, Caleb Mackay Salpeter Irvine, Kathryn H. Jacobsen, Nader Jahanmehr, Mihajlo B. Jakovljevic, Simerjot K. Jassal, Mehdi Javanbakht, Sudha P. Jayaraman, Panniyammakal Jeemon, Paul N. Jensen, Vivekanand Jha, Guohong Jiang, Denny John, Sarah Charlotte Johnson, Catherine O. Johnson, Jost B. Jonas, Mikk Jürisson, Zubair Kabir, Rajendra Kadel, Amaha Kahsay, Ritul Kamal, Haidong Kan, Nadim E. Karam, André Karch, Corine Kakizi Karema, Amir Kasaeian, Getachew Mullu Kassa, Nigussie Assefa Kassaw, Nicholas J. Kassebaum, Anshul Kastor, Srinivasa Vittal Katikireddi, Anil Kaul, Norito Kawakami, Peter Njenga Keiyoro, Andre Pascal Kengne, Andre Keren, Yousef Saleh Khader, Ibrahim A. Khalil, Ejaz Ahmad Khan, Young-Ho Khang, Ardeshir Khosravi, Jagdish Khubchandani, Aliasghar Ahmad Kiadaliri, Christian Kieling, Yun Jin Kim, Daniel Kim, Pauline Kim, Ruth W. Kimokoti, Yohannes Kinfu, Adnan Kisa, Katarzyna A. Kissimova-Skarbek, Mika Kivimaki, Ann Kristin Knudsen, Yoshihiro Kokubo, Dhaval Kolte, Jacek A. Kopec, Soewarta Kosen, Parvaiz A. Koul, Ai Koyanagi, Michael Kravchenko, Sanjay Krishnaswami, Kristopher J. Krohn, G. Anil Kumar, Pushpendra Kumar, Sanjiv Kumar, Hmwe H. Kyu, Dharmesh Kumar Lal, Ratilal Lalloo, Nkurunziza Lambert, Qing Lan, Anders Larsson, Pablo M. Lavados, Janet L. Leasher, Paul H. Lee, Jong-Tae Lee, James Leigh, Cheru Tesema Leshargie, Janni Leung, Ricky Leung, Miriam Levi, Yichong Li, Yongmei Li, Darya Li Kappe, Xiaofeng Liang, Misgan Legesse Liben, Stephen S. Lim, Shai Linn, Patrick Y. Liu, Angela Liu, Shiwei Liu, Yang Liu, Rakesh Lodha, Giancarlo Logroscino, Stephanie J. London, Katharine J. Looker, Alan D.

Lopez, Stefan Lorkowski, Paulo A. Lotufo, Nicola Low, Rafael Lozano, Timothy C. D. Lucas, Erlyn Rachelle King Macarayan, Hassan Magdy Abd El Razek, Mohammed Magdy Abd El Razek, Mahdi Mahdavi, Marek Majdan, Reza Majdzadeh, Azeem Majeed, Reza Malekzadeh, Rajesh Malhotra, Deborah Carvalho Malta, Abdullah A. Mamun, Helena Manguerra, Treh Manhertz, Ana Mantilla, Lorenzo G. Mantovani, Chabila C. Mapoma, Laurie B. Marczak, Jose Martinez-Raga, Francisco Rogerlândio Martins-Melo, Ira Martopullo, Winfried März, Manu Raj Mathur, Mohsen Mazidi, Colm McAlinden, Madeline McGaughey, John J. McGrath, Martin McKee, Claire McNellan, Suresh Mehata, Man Mohan Mehndiratta, Tefera Chane Mekonnen, Peter Memiah, Ziad A. Memish, Walter Mendoza, Mubarek Abera Mengistie, Desalegn Tadese Mengistu, George A. Mensah, Tuomo J. Meretoja, Atte Meretoja, Haftay Berhane Mezgebe, Renata Micha, Anoushka Millear, Ted R. Miller, Edward J. Mills, Mojde Mirarefin, Erkin M. Mirrakhimov, Awoke Misganaw, Shiva Raj Mishra, Philip B. Mitchell, Karzan Abdulmuhsin Mohammad, Alireza Mohammadi, Kedir Endris Mohammed, Shafiu Mohammed, Sanjay K. Mohanty, Ali H. Mokdad, Sarah K. Mollenkopf, Lorenzo Monasta, Marcella Montico, Maziar Moradi-Lakeh, Paula Moraga, Rintaro Mori, Chloe Morozoff, Shane D. Morrison, Mark Moses, Cliff Mountjoy-Venning, Kalayu Birhane Mruts, Ulrich O. Mueller, Kate Muller, Michele E. Murdoch, Gudlavalleti Venkata Satyanarayana Murthy, Kamarul Imran Musa, Jean B. Nachega, Gabriele Nagel, Mohsen Naghavi, Aliya Naheed, Kovin S. Naidoo, Luigi Naldi, Vinay Nangia, Gopalakrishnan Natarajan, Dumessa Edessa Negasa, Ruxandra Irina Negoi, Ionut Negoi, Charles R. Newton, Josephine Wanjiku Ngunjiri, Trang Huyen Nguyen, Quyen Le Nguyen, Cuong Tat Nguyen, Grant Nguyen, Minh Nguyen, Emma Nichols, Dina Nur Anggraini Ningrum, Sandra Nolte, Vuong Minh Nong, Bo Norrving, Jean Jacques N. Noubiap, Martin J. O'Donnell, Felix Akpojene Ogbo, In-Hwan Oh, Anselm Okoro, Olanrewaju Oladimeji, Tinuke Oluwasefunmi Olagunju, Andrew Toyin Olagunju, Helen E. Olsen, Bolajoko Olubukunola Olusanya, Jacob Olusegun Olusanya, Kanyin Ong, John Nelson Opio, Eyal Oren, Alberto Ortiz, Aaron Osgood-Zimmerman, Majdi Osman, Mayowa O. Owolabi, Mahesh Pa, Rosana E. Pacella, Adrian Pana, Basant Kumar Panda, Christina Papachristou, Eun-Kee Park, Charles D. Parry, Mahboubeh Parsaeian, Scott B. Patten, George C. Patton, Katherine Paulson, Neil Pearce, David M. Pereira, Norberto Perico, Konrad Pesudovs, Carrie Beth Peterson, Max Petzold, Michael Robert Phillips, David M. Pigott, Julian David Pillay, Christine Pinho, Dietrich Plass, Martin A. Pletcher, Svetlana Popova, Richie G. Poulton, Farshad Pourmalek, Dorairaj Prabhakaran, Noela M. Prasad, Narayan Prasad, Carrie Purcell, Mostafa Qorbani, Reginald Quansah, Beatriz Paulina Ayala Quintanilla, Rynaz H. S. Rabiee, Amir Radfar, Anwar Rafay, Kazem Rahimi, Afarin Rahimi-Movaghar, Vafa Rahimi-Movaghar, Mohammad Hifz Ur Rahman, Mahfuzar Rahman, Rajesh Kumar Rai, Sasa Rajsic, Usha Ram, Chhabi Lal Ranabhat, Zane Rankin, Puja C. Rao, Paturi Vishnupriya Rao, Salman Rawaf, Sarah E. Ray, Robert C. Reiner, Nikolas Reinig, Marissa B. Reitsma, Giuseppe Remuzzi, Andre M. N. Renzaho, Serge Resnikoff, Satar Rezaei, Antonio L. Ribeiro, Luca Ronfani, Gholamreza Roshandel, Gregory A. Roth, Ambuj Roy, Enrico Rubagotti, George Mugambage Ruhago, Soheil Saadat, Nafis Sadat, Mahdi

Safdarian, Sare Safi, Saeid Safiri, Rajesh Sagar, Ramesh Sahathevan, Joseph Salama, Huda Omer Ba Saleem, Joshua A. Salomon, Sundeep Santosh Salvi, Abdallah M. Samy, Juan R. Sanabria, Damian Santomauro, Itamar S. Santos, João Vasco Santos, Milena M. Santric Milicevic, Benn Sartorius, Maheswar Satpathy, Monika Sawhney, Sonia Saxena, Maria Inês Schmidt, Ione J. C. Schneider, Ben Schöttker, David C. Schwebel, Falk Schwendicke, Soraya Seedat, Sadaf G. Sepanlou, Edson E. Servan-Mori, Tesfaye Setegn, Katya Anne Shackelford, Amira Shaheen, Masood Ali Shaikh, Mansour Shamsipour, Sheikh Mohammed Shariful Islam, Jayendra Sharma, Rajesh Sharma, Jun She, Peilin Shi, Chloe Shields, Girma Temam Shifa, Mika Shigematsu, Yukito Shinohara, Rahman Shiri, Reza Shirkoohi, Shreya Shirude, Kawkab Shishani, Mark G. Shrime, Abla Mehio Sibai, Inga Dora Sigfusdottir, Diego Augusto Santos Silva, João Pedro Silva, Dayane Gabriele Alves Silveira, Jasvinder A. Singh, Narinder Pal Singh, Dhirendra Narain Sinha, Eirini Skiadaresi, Vegard Skirbekk, Erica Leigh Slepak, Amber Sligar, David L. Smith, Mari Smith, Badr H. A. Sobaih, Eugene Sobngwi, Reed J. D. Sorensen, Tatiane Cristina Moraes Sousa, Luciano A. Sposato, Chandrashekhar T. Sreeramareddy, Vinay Srinivasan, Jeffrey D. Stanaway, Vasiliki Stathopoulou, Nicholas Steel, Murray B. Stein, Dan J. Stein, Timothy J. Steiner, Caitlyn Steiner, Sabine Steinke, Mark Andrew Stokes, Lars Jacob Stovner, Bryan Strub, Michelle Subart, Muawiyyah Babale Sufiyan, Bruno F. Sunguya, Patrick J. Sur, Soumya Swaminathan, Bryan L. Sykes, Dillon O. Sylte, Rafael Tabarés-Seisdedos, Getachew Redae Taffere, Jukka S. Takala, Nikhil Tandon, Mohammad Tavakkoli, Nuno Taveira, Hugh R. Taylor, Arash Tehrani-Banihashemi, Tesfalidet Tekelab, Abdullah Sulieman Terkawi, Dawit Jember Tesfaye, Belay Tesssema, Ornwipa Thamsuwan, Katie E. Thomas, Amanda G. Thrift, Tenaw Yimer Tiruye, Ruoyan Tobe-Gai, Mette C. Tollanes, Marcello Tonelli, Roman Topor-Madry, Miguel Tortajada, Mathilde Touvier, Bach Xuan Tran, Suryakant Tripathi, Christopher Troeger, Thomas Truelsen, Derrick Tsoi, Kald Beshir Tuem, Emin Murat Tuzcu, Stefanos Tyrovolas, Kingsley N. Ukwaja, Eduardo A. Undurraga, Chigozie Jesse Uneke, Rachel Updike, Olalekan A. Uthman, Benjamin S. Chudi Uzochukwu, Job F. M. van Boven, Santosh Varughese, Tommi Vasankari, S. Venkatesh, Narayanaswamy Venketasubramanian, Ramesh Vidavalur, Francesco S. Violante, Sergey K. Vladimirov, Vasiliy Victorovich Vlassov, Stein Emil Vollset, Fiseha Wadilo, Tolassa Wakayo, Yuan-Pang Wang, Marcia Weaver, Scott Weichenthal, Elisabete Weiderpass, Robert G. Weintraub, Andrea Werdecker, Ronny Westerman, Harvey A. Whiteford, Tissa Wijeratne, Charles Shey Wiysonge, Charles D. A. Wolfe, Rachel Woodbrook, Anthony D. Woolf, Abdulhalik Workicho, Denis Xavier, Gelin Xu, Simon Yadgir, Mohsen Yaghoubi, Bereket Yakob, Lijing L. Yan, Yuichiro Yano, Pengpeng Ye, Hassen Hamid Yimam, Paul Yip, Naohiro Yonemoto, Seok-Jun Yoon, Marcel Yotebieng, Mustafa Z. Younis, Zoubida Zaidi, Maysaa El Sayed Zaki, Elias Asfaw Zegeye, Zerihun Menlkalew Zenebe, Xueying Zhang, Maigeng Zhou, Ben Zipkin, Sanjay Zodpey, Liesl Joanna Zuhlke, and Christopher J. L. Murray. 2017. 'Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016', *The Lancet*, 390: 1211-59.

Walker, C. S., P. J. Brockie, D. M. Madsen, M. M. Francis, Y. Zheng, S. Koduri, J. E. Mellem, N. Strutz-Seebohm, and A. V. Maricq. 2006. 'Reconstitution of invertebrate glutamate receptor function depends on stargazin-like proteins', *Proceedings of the National Academy of Sciences of the United States of America*, 103: 10781-86.

Wang, H., M. Yu, M. Ochani, C. A. Amella, M. Tanovic, S. Susarla, J. H. Li, H. Wang, H. Yang, L. Ulloa, Y. Al-Abed, C. J. Czura, and K. J. Tracey. 2003. 'Nicotinic acetylcholine receptor α7 subunit is an essential regulator of inflammation', *Nature*, 421: 384-88.

Wang, R., J. E. Mellem, M. Jensen, P. J. Brockie, C. S. Walker, F. J. Hoerndli, L. Hauth, D. M. Madsen, and A. V. Maricq. 2012. 'The SOL-2/Neto auxiliary protein modulates the function of AMPA-subtype ionotropic glutamate receptors', *Neuron*, 75: 838-50.

Wang, R., C. S. Walker, P. J. Brockie, M. M. Francis, J. E. Mellem, D. M. Madsen, and A. V. Maricq. 2008. 'Evolutionary conserved role for TARPs in the gating of glutamate receptors and tuning of synaptic function', *Neuron*, 59: 997-1008.

Wei, D. X., W. Y. Yang, S. Q. Huang, Y. F. Lu, T. C. Su, J. H. Ma, W. X. Hu, and N. F. Xie. 1981. 'Parasitological investigation on the ancient corpse of the Western Han Dynasty unearthed from tomb No. 168 on Phoenix Hill in Jiangling county', *Acta Acad Med Wuhan*, 1: 16-23.

Weiland, S., D. Bertrand, and S. Leonard. 2000. 'Neuronal nicotinic acetylcholine receptors: from the gene to the disease', *Behavioural Brain Research*, 113: 43-56.

Weisblat, D. A., and R. L. Russell. 1976. 'Propagation of electrical activity in the nerve cord and muscle syncytium of the nematodeAscaris lumbricoides', *Journal of comparative physiology*, 107: 293-307.

Wessler, Ignaz, Heinz Kilbinger, Fernando Bittinger, Ronald Unger, and Charles James Kirkpatrick. 2003. 'The non-neuronal cholinergic system in humans: Expression, function and pathophysiology', *Life Sciences*, 72: 2055-61.

White, J. 1988. *The Anatomy* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York).

White, J. G., E. Southgate, J. N. Thomson, and S. Brenner. 1986. 'The structure of the nervous system of the nematode Caenorhabditis elegans', *Philosophical Transactions of the Royal Society of London. B, Biological Sciences*, 314: 1-340.

WHO. 2005. 'National Policy on Traditional Medicine and Regulation of Herbal Medicines- Report of a WHO Global Survey.', Geneva, Switzerland: WHO. http://apps.who.int/medicinedocs/en/d/Js7916e/.

———. 2017a. *Preventive chemotherapy to control soil-transmitted helminth infections in at-risk population groups*.

———. 2017b. 'Summary of global update on preventive chemotherapy implementation in 2016: Crossing the billion', *Wkly. Epidemiol. Rec.*, 92: 20.

———. 2019. 'Soil-transmitted helminth infections'. http://www.who.int/news-room/fact-sheets/detail/soil-transmitted-helminth-infections.

Williamson, S. M., A. P. Robertson, L. Brown, T. Williams, D. J. Woods, R. J. Martin, D. B. Sattelle, and A. J. Wolstenholme. 2009. 'The Nicotinic Acetylcholine Receptors of the Parasitic Nematode Ascaris suum: Formation of Two Distinct Drug Targets by Varying the Relative Expression Levels of Two Subunits', *PLOS Pathogens*, 5: e1000517.

Wolstenholme, A. J., and A. T. Rogers. 2005. 'Glutamate-gated chloride channels and the mode of action of the avermectin/milbemycin anthelmintics', *Parasitology*, 131 Suppl: S85-95.

Wolstenholme, Adrian J., Ian Fairweather, Roger Prichard, Georg von Samson-Himmelstjerna, and Nicholas C. Sangster. 2004. 'Drug resistance in veterinary helminths', *Trends in Parasitology*, 20: 469-76.

Wu, Jie, Qiang Liu, Pei Tang, Jens D. Mikkelsen, Jianxin Shen, Paul Whiteaker, and Jerrel L. Yakel. 2016. 'Heteromeric α7β2 Nicotinic Acetylcholine Receptors in the Brain', *Trends in Pharmacological Sciences*, 37: 562-74.

Wu, X., H. Chen, T. Gan, J. Chen, C. Ngo, and Q. Peng. 2016. 'Automatic Hookworm Detection in Wireless Capsule Endoscopy Images', *IEEE Transactions on Medical Imaging*, 35: 1741-52.

Yan, D., and S. Tomita. 2012. 'Defined criteria for auxiliary subunits of glutamate receptors', *The Journal of Physiology*, 590: 21-31.

Yassin, L., B. Gillo, T. Kahan, S. Halevi, M. Eshel, and M. Treinin. 2001. 'Characterization of the DEG-3/DES-2 Receptor: A Nicotinic Acetylcholine Receptor That Mutates to Cause Neuronal Degeneration', *Molecular and Cellular Neuroscience*, 17: 589-99.

Zhao, L., Y.-P. Kuo, A. A. George, J.-H. Peng, M. S. Purandare, K. M. Schroeder, R. J. Lukas, and J. Wu. 2003. 'Functional Properties of Homomeric, Human α7-Nicotinic Acetylcholine Receptors Heterologously Expressed in the SH-EP1 Human Epithelial Cell Line', *Journal of Pharmacology and Experimental Therapeutics*, 305: 1132.

Zheng, Y., J. E. Mellem, P. J. Brockie, D. M. Madsen, and A. V. Maricq. 2004. 'SOL-1 is a CUB-domain protein required for GLR-1 glutamate receptor function in C. elegans', *Nature*, 427: 451-57.

Ziem, J. B., I. M. Kettenis, A. Bayita, E. A. Brienen, S. Dittoh, J. Horton, A. Olsen, P. Magnussen, and A. M. Polderman. 2004. 'The short-term impact of albendazole treatment on Oesophagostomum bifurcum and hookworm infections in northern Ghana', *Ann Trop Med Parasitol*, 98: 385-90.

Ziem, J. B., A. Olsen, P. Magnussen, J. Horton, E. Agongo, R. B. Geskus, and A. M. Polderman. 2006. 'Distribution and clustering of Oesophagostomum bifurcum and hookworm infections in Northern Ghana', *Parasitology*, 132: 525-34.

Ziff, E. B. 2007. 'TARPs and the AMPA receptor trafficking paradox', *Neuron*, 53: 627-33.

Zoli, Michele, Francesco Pistillo, and Cecilia Gotti. 2015. 'Diversity of native nicotinic receptor subtypes in mammalian brain', *Neuropharmacology*, 96: 302-11.

APPENDIX. ANTHELMINTICS: THE BEST WAY TO PREDICT THE FUTURE IS TO CREATE IT

A paper published in the *Veterinary Parasitology* (2015)[1]

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A1.0 Abstract

'The best way to predict the future is to create it.' When we look at drugs that are used to control parasites, we see that new knowledge has been created (discovered) about their modes of action. This knowledge will allow us to predict combinations of drugs which can be used together rationally to increase the spectrum of action and to slow the development of anthelmintic resistance. In this paper we comment on some recent observations of ours on the modes of action of emodepside, diethylcarbamazine and tribendimidine. Emodepside increases the activation of a SLO-1 K+ current inhibiting movement, and diethylcarbamazine has a synergistic effect on the effect of emodepside on the SLO-1 K+ current, increasing the size of the response. The combination may be considered for further testing for therapeutic use. Tribendimidine is a selective cholinergic nematode B-subtype nAChR agonist, producing muscle depolarization and contraction. It has different subtype selectivity to levamisole and may be effective in the presence of some types of levamisole resistance. The new information about the modes of action may aid the design of rational drug combinations designed to slow the development of resistance or increase the spectrum of action.

A1.1 Keywords

Anthelmintic; mode of action; tribendimidine; emodepside; diethylcarbamazine

A2.0 Introduction

'The best way to predict the future is to create it.' This quote is usually ascribed to Abraham Lincoln and leads to some interesting thoughts when applied to different research fields including our own field of study, that is, anthelmintics. What are we creating and what can we see for the future for anti-parasitic drugs? We are creating new techniques for the screening of anthelmintic drugs, we are creating new methods for studying the modes of actions of anthelmintics and we are creating new ways for detecting anthelmintic resistance (Gilleard and Beech, 2007 and Lanusse et al., 2014). We are also creating better awareness of the 'neglected tropical diseases' (Hotez et al., 2008) of humans which include ascariasis, hookworm and trichuriasis and better awareness of the link between human and animal medicine, the 'One Health' concept. Our western economic system has also produced a more favorable economic environment for the development of animal anthelmintics, than anthelmintics for humans. Currently, we limit the damage done by nematode parasites of animals with pasture management, and improved targeted metaphylactic and therapeutic use of anthelmintics; for humans, clean water and sanitation are limiting factors (like clean pasture for animals) while mass drug administration (MDA) has similarities to the regular metaphylactic use of anthelmintics for animals. Vaccinations against parasites for both humans and animals are very desirable but so far they have limited efficacy.

The advances and creations listed above lead to a number of logical predictions for the future use and development of anthelmintics. We are confident that the

development and better understanding of anthelmintic properties will continue, at a steady but modest pace, driven by economic, human and animal needs. We think that the economic pressures associated with animal medicine will remain greater than for human medicine and focused on the development of novel ‘resistance-busting’ anthelmintics. Success in research can be limited by funding as was said by the father of modern drug development, Paul Ehrlich, Figure 1 (1854–1915), who recognized success required the four Gs: Gluck, Geduld, Geshcick und Geld (luck, patience, skill and money) (Perutz, 1988). The ‘money’ for animal anthelmintics comes from the market for anti-parasitic drugs and chemicals for small animals and livestock which was estimated at $11 billion (Evans and Chapple, 2002). This contrasts with the market for human anthelmintics which is only $0.5 billion despite some 2 billion humans being infected in developing countries, around 25 cents per person annually! Given that the out of pocket costs of new human drugs may be $403 million (at year 2000 valuations (DiMasi et al., 2003) or more, we can see that the commercial development of human anthelmintic drugs is not favorable. It seems more likely that the economics of animal health will drive the development and advance of the knowledge base of animal anthelmintics and that these developments will be applied and adapted for human use (ivermectin and perhaps emodepside) unless private charities, governments and foundations overcome the financial limitations. In addition to the gradual development of resistance-busting anthelmintics we see: developments in our understanding of the modes of action of anthelmintics; we see more logical combinations of anthelmintics to slow down or

Fig. 1. Paul Erlich in his Frankfurt office, circa 1900, the father of modern chemotherapy, who worked on trypanosome diseases and popularized the concept of the ‘magic bullet’ (magische Kugel, the perfect therapeutic agent).

counter the development of resistance and; also new methods for detecting anthelmintic resistance.

Our lab has focused on understanding of the modes of action of anthelmintics and in this paper we illustrate some of our recent observations and developments in our understanding of the actions of emodepside, diethylcarbamazine and tribendimidine. The mode of action of these anthelmintics involves effects on membrane ion-channels and has required us to use electrophysiological techniques for their study (Martin et al., 1996b). We think that better knowledge of the mode of action of these compounds will allow rational combination with other anthelmintics to increase potency, spectra and allow a slowing of the speed of development of resistance in animal and human parasites. This paper is based on a lecture given to the World Association for the Advancement of Veterinary Parasitology (WAAVP) in Liverpool, 2015 and covers: (1) emodepside, an anthelmintic used for small animals, which has the potential for being used for human use to control filarial parasites; (2) diethylcarbamazine, a long serving anthelmintic still used

for the control of filariasis in humans and; (3) tribendimidine, a recent anthelmintic developed by China for human use.

A3.0 Emodepside

Emodepside is a cyclooctadepsipeptide developed by Bayer, Fig. 2, which is related to its parent compound, PF1022A (Martin et al., 1996a) which has broad-spectrum anthelmintic activity. Emodepside has an inhibitory effect on locomotion that allowed (Guest et al., 2007) to use a *C. elegans* mutagenesis screen to find that slo-1 (a Ca^{2+}-dependent K^{+} channel) mutant alleles were resistant to the inhibitory effects of emodepside. Crisford et al. (2011) also described transgenic experiments in which *C. elegans* SLO-1a channels were swapped for KCNMA1, the human orthologue of SLO-1 channels. These studies found that the sensitivity to emodepside in the rescues depended upon the origin of the SLO-1 channel: the human KCNMA1 channel was 10–100 times less sensitive to emodepside than the rescues expressing *C. elegans* SLO-1a channel. These experiments suggested that SLO-1 Ca^{2+}-dependent K^{+} channels of nematodes are a major part of the target site of emodepside. Expression of *C. elegans* SLO-1a channels in *Xenopus* oocytes have revealed that emodepside can directly open these channels (Kulke et al., 2014) and that emodepside action on this splice variant of the channel does not require the presence of additional receptors like the latrophilin receptors for an emodepside effect (Willson et al., 2004). It does not however, rule out a contribution of latrophilin receptors to the overall mode of action of emodepside in parasites as indicated by the interaction of Lat-1 and emodepside (Saeger et al., 2001).

SLO-1 K_+ channels have large (~200 pS) single-channel conductances and are also known as 'big' potassium (BK) channels. These channels serve to clamp the membrane potential near the potassium reversal potential and inhibit electrical excitability. The SLO-1 K_+ channel, has a different function to the high conductance Ca_{2+}-dependent Cl_- channel (Thorn and Martin, 1987) which is associated with the transport of carboxylic acids from anaerobic metabolism of glucose (Valkanov and Martin, 1995). Subunits homologous to the vertebrate SLO-1 K_+ channels are found in C. elegans and *Ascaris suum* (Buxton et al., 2011). The SLO-1 K_+ channels are composed of 4 α-subunits; α-subunits have seven (S0–S6) transmembrane regions, a P-loop between S5 and S6, a large intracellular domain (S7-S-10) and a well conserved 'calcium bowl' between domains S9 and S10, Fig. 2. In addition, the regulator of the K_+ channel conductance (RCK) domains (S7, S8) contains high and low affinity calcium binding sites. The SLO-1 α-subunits show alternative splicing, producing channels with different calcium sensitivities; in *C. elegans* there are up to 15 splice variants (including SLO-1a, SLO-1b and SLO-1c (Kulke et al., 2014 and Wang et al., 2001)). Each α-subunit of the channel has at least two high affinity calcium binding sites and one low affinity calcium/magnesium binding site. The channel also combines with secondary, regulatory β-subunits in vertebrates (Knaus et al., 1994) but these subunits have not yet been identified in *C. elegans*. However, *Drosophila*, which also lacks β subunits, has SLOb proteins, which appear to carry out similar functions to the vertebrate β-subunits and these may be present in nematodes (Claridge-Chang et al., 2001).

The suggested function of the SLO-1 K_+ channels is that they adjust the resting

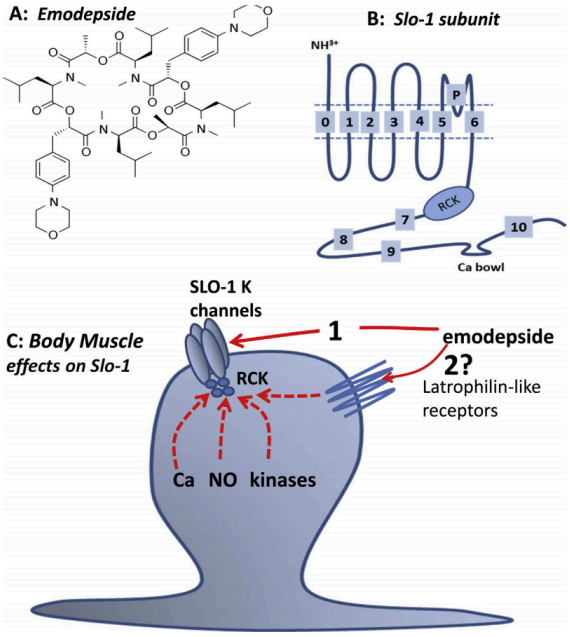

Fig. 2. Summary diagrams of emodepside structure, SLO-1 subunit and a model of the mode action of emodepside on nematode body muscle. A: Emodepside. B: Line diagram of structure of one SLO-1 subunit; each SLO-1 K+ channel is made up of 4 of these subunits. C. Putative mode of action of emodepside on SLO- 1K+ channels in the muscle: act directly (1) or indirectly by stimulating latrophilin-like receptors (2) and signaling cascades that may involve NO, protein Kinase C and/or calcium. It is unlikely that emodepside acts at the extracellular surface of the SLO-1 K+ channel because of the slow time course of its action. It is very lipophilic and could act in the lipid membrane phase on the SLO-1 K+ channel or move into the cytoplasm and act intracellularly. A SLO-1 K+ channel (C) is shown composed of 4 subunits along with the 'RCK' cytoplasmic regulatory region of the channel. (Martin et al., 2012).

membrane potential of electrically excitable cells and adjust the level of excitability, up or down, and so affect the response to other inputs. The opening of the SLO-1 K_+ channel, Fig. 2, is regulated by at least ten factors including: (1) membrane potential; (2) calcium; (3) magnesium; (4) NO; (5) CO; (6) arachidonic acid; (7) prostaglandins; (8) phosphorylation by cAMP-dependent protein kinase A; (9) phosphorylation by diacylgycerol/Ca_{2+}-dependent protein kinase C; and (10) phosphorylation by cGMP-dependent protein kinase G (Salkoff et al., 2006). The kinases allow SLO-1 to be coupled to multiple and quite diverse signaling cascades permitting different ways of adjusting the excitability of the cells. The different nematode neuropeptides could affect cAMP and

cGMP levels (e.g., AF1, AP2, and PF1 and PF2) could then affect SLO-1 channels (Muhlfeld et al., 2009, Verma et al., 2007 and Verma et al., 2009). A selective agonist for the PF1 and/or a selective antagonist of the AF1 receptor would activate a SLO-1 K_+-like current and increase the efficacy of emodepside. The neuropeptides AF1, AF10 and PF2 also bind with low affinity to the latrophilin-like receptor, HC110-R of *Haemonchus contortus*, suggesting that emodepside could also have an indirect mode of action on SLO-1 K_+ channels through these latrophilin-like receptors (Fig. 2) that are neuropeptide receptors (Muhlfeld et al., 2009).

We found (Buxton et al., 2011 and Martin et al., 2012) that *Asu-slo-1* is an evolutionarily conserved homologue of the *slo-1* genes, expressed in adult *A. suum* body muscle flaps. Using a two-micro-electrode current-clamp and voltage-clamp technique, Fig. 3A, we found in the nematode parasite *A. suum*, that emodepside activates SLO-1-like K_+ channels to produce hyperpolarization under current-clamp, Fig. 3B1, and an outward current when under voltage-clamp, Fig. 3B2. We also found that emodepside increased voltage activated K_+ currents, Fig. 3C and D, in a time dependent manner. Emodepside, Fig. 3C, unlike PF1 did not decrease calcium currents and so emodepside does not work by releasing PF1 as has been hypothesized. We found that the effects of emodepside on the voltage activated K_+ channels is Ca_{2+}-dependent and were inhibited by 5 mM 4-aminopyridine, Fig. 4A. The membrane hyperpolarization and increase in voltage-activated K_+ current produced by emodepside, Fig. 3B, are very slow in onset and increase over a period of more than 10 min (Buxton et al., 2011); the slow onset effect of emodepside might be due to its very lipophilic nature and a membrane partitioning effect. It may also be because the effects of emodepside are indirect and produced by activation

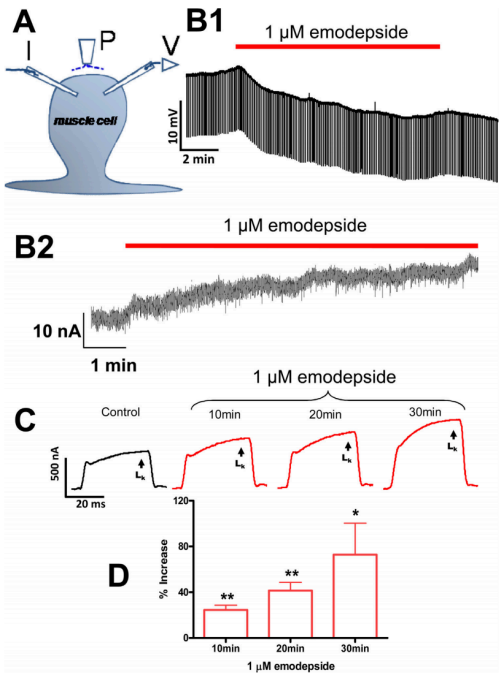

Fig. 3. Electrophysiological techniques (two micropipette current-clamp and voltage-clamps) for recording from *Ascaris suum*. A: *A. suum* muscle bag showing the current (I) and voltage (V) micropipettes in the bag, and the perfusion needle (P). B1: Representative current-clamp trace showing the slow hyperpolarizing membrane potential during and after 10 min application of 1 µM emodepside. B2: Outward current response to 1 µM emodepside at higher time resolution. Holding potential −35 mV. Notice that emodepside produces a gradually increasing current after a delay of some 30 seconds. The response does not plateau in the time period of this recording. C: Voltage-clamp traces of control K+ current and the time-dependent effects of 1 µM emodepside on the K+ currents, all to a step potential of 0 mV from a holding potential of −35 mV. D: Bar chart (mean ± S.E.) of 1 µM emodepside effect on steady state (LK) currents. Comparison was made between the control 0 mV step current at 30−40 ms and the corresponding current increased by emodepside at 10, 20 and 30 min. Emodepside increased LK currents at 10 min ($p < 0.01$, $n = 4$, paired t-test), 20 min ($p < 0.01$, $n = 4$, paired t-test) and 30 min ($p < 0.05$, $n = 4$, paired t-test). (Martin et al., 2012).

of a slow signaling cascade. Fig. 4B and C shows that the effects of emodepside are

potentiated by sodium nitroprusside (a NO donor), antagonized by iNOS inhibitors

(NNLA), and inhibited by 1 µM staurosporine, an inhibitor of protein kinase (Buxton et

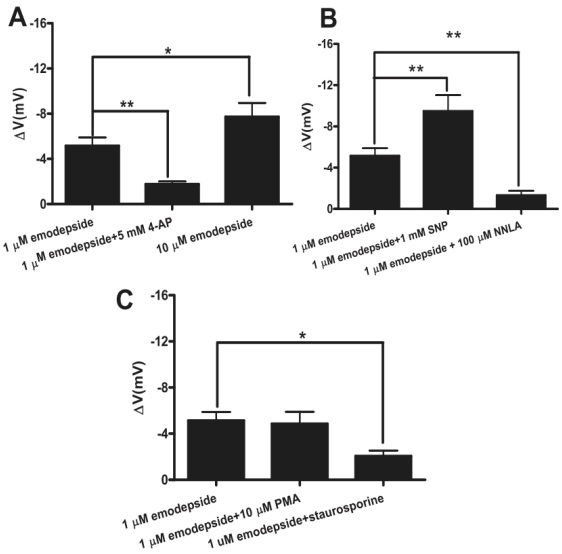

Fig. 4. Effect of 1 μM emodepside on membrane potential. A: Comparison was made between membrane potential before and during emodepside application. 1 μM emodepside caused a significant membrane hyperpolarization ($p < 0.001$, $n = 10$, paired t-test) which was reduced in the presence of 5 mM 4-aminopyridine ($p < 0.01$, $n = 8$, unpaired t-test). 10 μM emodepside caused an increased hyperpolarization in comparison to 1 μM emodepside ($p < 0.05$, unpaired t-test). B: Bar chart (mean ± s.e) of NO influence on emodepside-induced hyperpolarization. In the presence of 1 mM SNP, 1 μM emodepside caused an increased hyperpolarization ($p < 0.01$, $n = 4$, paired t-test). 100 μM NNLA decreased the hyperpolarization caused by 1 μM emodepside ($p < 0.01$, unpaired t-test). C: Bar chart (mean ± s.e) of effect of protein kinase modulators on emodepside induced hyperpolarization. 10 μM PMA had no significant effects on the hyperpolarization caused by 1 μM emodepside. However, 1 μM staurosporine decreased the hyperpolarization caused by 1 μM emodepside ($p < 0.05$, unpaired t-test). (Martin et al., 2012).

al., 2011). Interestingly, these signaling molecules are known as activators of SLO-1 in other cells (Bolotina et al., 1994b, Holden-Dye et al., 2007, Mistry and Garland, 1998 and Wang et al., 1999) and therefore encourage the view that emodepside could act through either or both of these signaling cascades and the signaling cascades may be in series or parallel, Fig. 2C. A number of studies on the mammalian orthologues of SLO-1 show that they are directly and alternately regulated by complex, multiple signaling cascades, involving NO and diacylglycerol or PKC activation (Ghatta et al., 2006 and

Salkoff et al., 2006). It is pointed out that NO signaling pathways in mammals may not be conserved in all species of nematode.

Thus emodepside appears to selectively activate the SLO-1 K_+ current that is present in nematodes but has less effect on the SLO-1 K_+ currents of vertebrates. This can explain the selective toxicity of the drug. The existence and mechanisms of resistance to emodepside in nematode parasites remain to be studied but could involve several different mechanisms including changes in the splice forms of the SLO-1 K_+ channel or its regulation by different signaling cascades. However, such resistance, depending on the specific mechanism, might be countered (Verma et al., 2007 and Verma et al., 2009) by a selective agonist for the FMRFamide PF1 receptor and/or a selective antagonist of the FMRFamide AF1 receptor which would activate a SLO-1 K_+-like current and increase the efficacy of emodepside. An increased knowledge of the physiology of target site of emodepside provides a new way of designing drug combinations. The complex nature of SLO-1 K_+ regulation suggests that if there were resistance to emodepside it would be complex in nature and therefore likely to be polygenic involving multiple mechanisms.

A4.0 Diethylcarbamazine

Diethylcarbamazine, Fig. 5A, is an antifilarial drug that has been used since 1947 against lymphatic filariasis and loiasis. It is still an important and effective antifilarial drug but its mode of action is not fully described. Diethylcarbamazine has been suggested to have an indirect, host mediated mode of action: it appears to alter host arachidonic acid, nitric oxide metabolic pathways and inhibits NF-κB, which together in an unknown way leads to immobilization and sequestration of the microfilariae (Maizels and Denham,

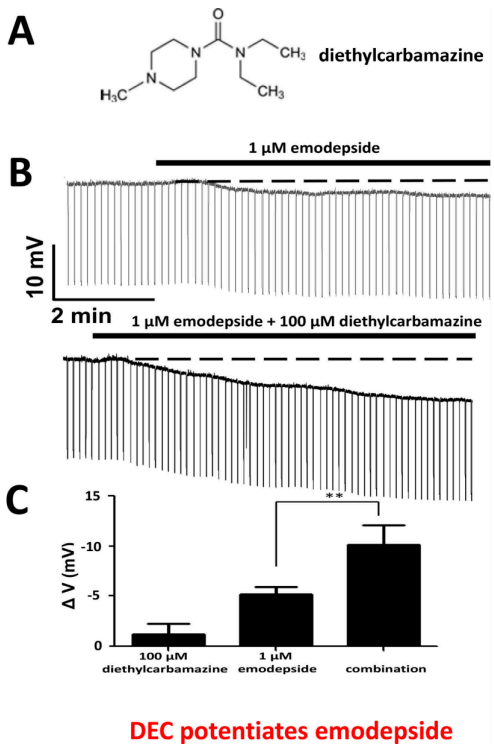

Fig 5. The combined effect of emodepside and diethylcarbamazine is greater than the effect of emodepside alone on *A. suum* membrane potential. A: Chemical structure of diethylcarbamazine. B: Representative current-clamp traces showing the membrane potential before, during 1 µM emodepside application (top trace) and during application of 1 µM emodepside plus 100 µM diethylcarbamazine (lower trace). The time delay between the end of the application of 100 µM diethylcarbamazine and 1 µM emodepside plus 100 µM diethylcarbamazine was 10 min. The change in membrane potential between the beginning of the 100 µM diethylcarbamazine and application of 1 µM emodepside plus 100 µM diethylcarbamazine was a hyperpolarization of 1.5 mV. C: Bar chart (mean ± i.e.) of diethylcarbamazine effect on emodepside-induced hyperpolarization. 100 µM diethylcarbamazine increased the hyperpolarization caused by 1 µM emodepside ($p < 0.01$, $n = 5$, unpaired t-test. (Buxton et al., 2014a).

1992 and Peixoto and Silva, 2014). Diethylcarbamazine activity against *B. malayi* microfilariae is abolished in inducible nitric oxide synthase knockout mice (iNOS$^{-/-}$), suggesting that diethylcarbamazine activity is dependent on host inducible nitric oxide synthase (iNOS) and nitric oxide (McGarry et al., 2005).

We were interested to determine how diethylcarbamazine would affect calcium dependent SLO-1 K^+ currents in isolated *A. suum* muscle flap preparations, and how

diethylcarbamazine interacts with emodepside. The interest was prompted by observations in vertebrates (Bolotina et al., 1994a) which show that nitric oxide activates SLO-1 K_+ channels and observations on *A. suum* indicating the presence of nitric oxide synthase and of SLO-1 K_+ channels which show positive modulation by a nitric oxide pathway (Buxton et al., 2011). We hypothesized that diethylcarbamazine, with effects on arachidonic acid and nitric oxide pathways, would increase activation of SLO-1 K_+ currents in *A. suum* muscle and potentiate effects of emodepside on membrane potential (Buxton et al., 2014a). We conducted current-clamp and voltage-clamp electrophysiological experiments, using *A. suum* body muscle in the presence of sufficient calcium to allow activation of the SLO-1 K_+ currents. We found that indeed diethylcarbamazine, by itself, can increase activation of SLO-1 K_+ currents and potentiate the hyperpolarizing effects of emodepside. This to us was very interesting since it showed that diethylcarbamazine has a direct effect on the nematode parasite and its effects are not exclusively mediated via the host as has been suggest by earlier experiments. It seems likely that the effects of diethylcarbamazine may involve effects on NO or arachidonic acid metabolites both in the host and in the parasite. Although the mechanism of action of diethylcarbamazine remains to be further defined, the synergistic effect of emodepside and diethylcarbamazine suggests that the combination of the two drugs could be considered for therapeutic use for the treatment of filarial nematode infections.

A5.0 Tribendimidine

Tribendimidine, Fig. 6, is a symmetrical diamidine derivative of amidantel which was developed in China for use in humans in the mid-1980s. It is a broad-spectrum

anthelmintic effective against soil-transmitted helminthiasis including hookworm, pinworms, roundworms, *Strongyloides* and flatworms of humans (Xiao et al., 2005 and Xu et al., 2014). Molecular studies (Hu et al., 2009) on *C. elegans* using null-mutants of the levamisole receptor subunits strongly suggested that tribendimidine is a cholinergic agonist that is selective for the same nematode muscle nAChR as levamisole. When we looked at these studies we found that there are no direct electrophysiological observations in nematode parasites that had been made to test this hypothesis and we were aware that sometimes observations on the model Clade V nematode were not always the same in parasitic nematodes, particularly for the parasites from a different Clade. We were also interested in trying to explain why tribendimidine is effective against some nematode parasites when levamisole is not.

We tested the effects of tribendimidine on the electrophysiology, Fig. 6, and contraction of *A. suum* muscle (Robertson et al., 2015) and found that tribendimidine produces a dose-dependent depolarization associated with an increase in the conductance of the muscle membrane as the nicotinic receptors (nAChRs) open. We found that tribendimidine was more potent than acetylcholine and had an EC_{50} of 0.8 μM. We also tested the effects of the nicotinic antagonist mecamylamine, a potent antagonist of muscle nAChRs of parasitic nematodes and found that 3 μM was potent against tribendimidine; the antagonist effects of mecamylamine confirmed the cholinergic action of tribendimidine on the nAChRs of the parasite *A. suum.*

We characterized the pharmacological profile of tribendimidine using our *Ascaris* muscle contraction assay systems (Qian et al., 2006 and Robertson et al., 2002) with tribendimidine as the agonist and methyllycaconitine, paraherquamide and derquantel as

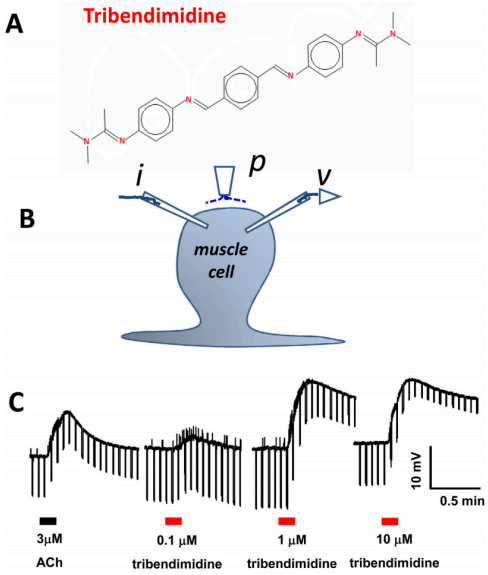

Fig. 6. A: Chemical structure of tribendimidine. B: Diagram of the two-micropipette current-clamp technique used to record the membrane potential (v) and to inject 40 nA hyperpolarizing 500 ms current pulses (i) at 0.3 Hz. p is the microperfusion pipette used to apply and wash off the drugs. C: Application of 3 μM acetylcholine and then 0.1, 1 and 10 μM tribendimidine to the same preparation. 1 μM tribendimidine produces a bigger depolarization response (upward movement) and conductance increase (reduction in the voltage responses to current injection, producing a narrowing of the width of the trace) than 3 μM acetylcholine (Robertson et al., 2015).

antagonists to calculate the potency, pA_2, of the antagonists and the subtype of the selectivity of tribendimidine. We found that tribendimidine was more selective for the **B-**subytpe (bephenium preferring) nAChRs than the **L-**subtype (levamisole preferring) or the **N-**subtype (nicotine preferring). These observations showed the selectivity for the different groups of nAChRs present on the *Ascaris* muscle was not the same as levamisole and it was possible that levamisole-resistant parasites would remain sensitive to tribendimidine. To test this we used, *Oesophagostomum dentatum* L_3 larval migration inhibition assays with levamisole sensitive isolates and levamisole-resistant isolates and tested the effects of tribendimidine on motility. Levamisole was less effective ($p < 0.001$, F-test) in inhibiting migration of levamisole-resistant larvae than the migration of levamisole-sensitive larvae. We tested the effects of tribendimidine to the limits of its

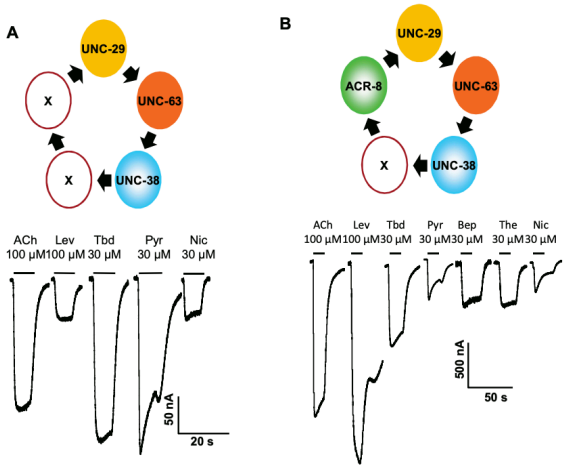

Fig. 7. Tribendimidine as an agonist of Ode-(29-8-38-63) and Ode-(29-63-38) receptors expressed in oocytes, currents recorded under voltage-clamp at −60 mV (Buxton et al., 2014b) A: Tribendimidine and pyrantel at 100 µM is more potent than levamisole on the Ode-(29-38-63) receptor. B: Levamisole at 100 µM is more potent than acetylcholine tribendimidine, pyrantel and nicotine on the expressed Ode-(29-8-38-63) receptor. Note that derquantel produces competitive antagonism of the effects of levamisole but a non-competitive antagonism of pyrantel, suggesting that the mode of action of levamisole and pyrantel are not identical.

solubility, ~30 µM, and found that tribendimidine was actually more potent on the levamisole-resistant isolate than on the levamisole-sensitive isolates ($p < 0.001$, F-test). The larger effect of tribendimidine on the levamisole-resistant, than the levamisole-sensitive isolates at lower tribendimidine concentrations, supports the view that levamisole and tribendimidine do not activate identical nAChR receptor subtypes. The higher efficacy of tribendimidine on the levamisole resistant *O. dentatum* isolate might indicate a case of negative cross resistance (Miltsch et al., 2013).

We have been able to express, in *Xenopus* oocytes a range of different nAChR receptor subtypes derived from anthelmintic sensitive *Oesophagostomum dentatum* clones of their nAChR receptor subunits (Buxton et al., 2014b). Fig. 7 shows a diagram of the subunit composition of two nAChR subtypes expressed in oocytes, one composed of Ode-UNC-29:Ode-UNC-63:Ode-UNC-38, the other composed of Ode-UNC-29:Ode-UNC-63:Ode-UNC-38:Ode-ACR-8 subunits. The Ode-UNC-29:Ode-UNC-63:Ode-

UNC-38 receptor was more sensitive to tribendimidine and pyrantel but not to levamisole or nicotine. We can see that the receptor subunit arrangements affect their pharmacology and that not all cholinergic anthelmintics have the same selectivity.

Our understanding to date is that there are a range of pharmacologically different nAChR subtypes present in parasitic nematodes and the pharmacology of the receptor subtypes vary with the subunit composition of the receptor which varies with the tissue and species of the nematode parasite. There will therefore be cholinergic anthelmintics that act on different nAChR subtypes and it is therefore possible that there will not always be cross resistance between the cholinergic anthelmintics.

A6.0 Discussion

Diethylcarbamazine is used mostly for treatment of filariasis in humans but has effects against hookworm and ascariasis, intestinal nematode parasites (Meyrowitsch and Simonsen, 2001). In addition to the treatment of filariasis, diethylcarbamazine, as a single dose treatment, has modest effects on intestinal nematode parasite infections including ascariasis and trichuriasis but is more effective when combined with ivermectin or albendazole (Belizario et al., 2003). We have seen in our experiments that emodepside increases a Slo-1 K^+ current in the parasite and that diethylcarbamazine can potentiate the effect of emodepside. Both of these compounds have been used separately as single anthelmintics but we do not have data on their therapeutic effect when used in combination. This information is desirable to obtain and if therapeutic combination of emodepside and diethylcarbamazine is safe and more potent than either compound in

isolation, such synergism could slow the development of resistance and extend the spectrum of action of the therapy.

Levamisole and tribendimidine have different selectivities for nAChR subtypes. In *Ascaris* tribendimidine is more selective for the B-subtype rather than the L-subtype of nAChR. Tribendimidine is more potent on expressed *O. dentatum* receptor subtypes composed of Ode-UNC-29:Ode-UNC-63:Ode-UNC-38 subunits, than on subtypes composed of Ode-UNC-29:Ode-UNC-63:Ode-UNC-38:Ode-ACR-8 subunits (Buxton et al., 2014b). The difference in selectivity suggests that tribendimidine has the potential to be effective against nematode parasites that are not sensitive to levamisole, including those parasites that have developed resistance. A careful combination of different cholinergic anthelmintics has the potential to extend the spectrum of action of the drug treatment. Tribendimidine has an interesting and promising pharmacology and has the potential for single-dose MDA with its broad-spectrum of action. Although tribendimidine appears safe and has broad-spectrum activity, a large-scale clinical study is advocated to further verify human safety.

A7.0 Conclusion

We have been excited by our discovery of further details of the modes of action of the different anthelmintics that we work on and believe that the information will be useful for defining mechanisms of resistance to the anthelmintic drugs that we have studied. We also think that the improved understanding of their mechanisms of action will lead to better use of these compounds and logical design of synergistic combinations. We do not look back, but ***forward***, to the future.

A8.0 Funding

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